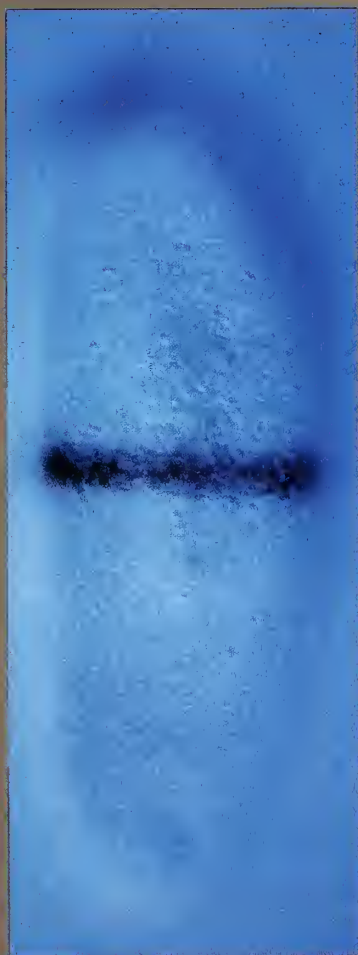


NATIONAL INSTITUTE OF CHILD HEALTH AND HUMAN DEVELOPMENT

INTRAMURAL RESEARCH PROGRAM

ANNUAL REPORT OF THE SCIENTIFIC DIRECTOR

1989



U.S. Department of Health and Human Services  
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#### Cover photo

Homeotic genes determine alternative morphologic pathways of the body parts. Mutations in these genes result in developmental anomalies, which can occur with changes in the embryonic genes *per se*, or in maternal genes, the products of which interact with genes in the progeny. The left panel demonstrates the product (wide band) of one homeotic gene (*Krüppel*) in a *Drosophila melanogaster* embryo, which is normally regulated by the product of a maternal gene (*fsh*). The central panel shows a dramatic decrease in the *Krüppel* product in an embryo whose mother carries a mutant *fsh* gene. The right hand panel shows a fly carrying a dominant mutation in another homeotic gene (*Antennapedia*), which results in transformation of the antennae into legs.

(Courtesy of Drs. Sue Haynes and Jim Kennison)

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# NATIONAL INSTITUTE OF CHILD HEALTH AND HUMAN DEVELOPMENT

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The Intramural Research Program is broadly concerned with the biological and neurobiological, medical, and behavioral aspects of normal and abnormal human development. In addition to four major clinical research and training programs in the areas of genetics and endocrinology, a diversity of developmental models are under study in twelve fundamental research Laboratories, drawing upon observations in bacteria, *Drosophila*, yeasts, viruses, molluscs, frogs, rodents, and subhuman primates. Disciplines employed in these studies include biochemistry, virology, molecular biology, immunology, pharmacology, genetics, cell and neuronal biology, biophysics, mathematical and theoretical biology, reproductive physiology, and comparative ethology. During the past year, we have emphasized a number of research programs that we believe have a particular promise and timeliness. In these areas, new resources have been added, so that the investigators will be optimally poised to exploit their recent findings. These programs address problems in the cellular and molecular biology of development (growth and differentiation), wherein we anticipate significant results with broad implications for biomedical research. Indeed, it is our notion that the central, and arguably the most exciting, question in contemporary biology is: What are the mechanisms by which gene expression is developmentally regulated?

The **Laboratory of Molecular Genetics (LMG)** continues to apply the tools of molecular biology to the study of a variety of aspects of gene transmission and control, particularly as they relate to embryonic development. Members of the LMG divide their efforts between integrative control systems, which are best studied in simple organisms such as viruses, bacteria and yeast, and the complex genetic control of differentiation in higher organisms.

One group of researchers in the LMG focuses on the  $\lambda$  phage family, partly because the genome of the *E. coli* bacteriophage,  $\lambda$ , is one of the best understood in molecular biology. Over past few years, this group has contributed much to our knowledge of mechanisms of gene control by studying termination and antitermination of RNA synthesis in  $\lambda$ . Recently, the group discovered how superinfection by  $\lambda$  of cells already infected with another phage of the  $\lambda$  family, HK022, is prevented. The product of HK022's *nun* gene prevents  $\lambda$ 's antitermination N protein from binding to the  $\lambda$  anti-termination (*nul*) site. This year the group established that *nun* is not under the control of the phage repressor (and therefore must lie upstream from the repressor) and that it bears about 85% sequence homology with the N protein. Other novel features of HK022 were also demonstrated. For example, transcription of the two intrinsically strong promoters, pL and pR, is prevented when DNA synthesis is inhibited. How these two processes, transcription and DNA synthesis, are linked is of great interest and the subject of ongoing investigations, because of the potential importance to the development of antiviral agents. In contrast to  $\lambda$ , transcription anti-termination in this phage does not require a specific phage encoded protein, and work is now under way to determine whether HK022 uses a host protein, or whether anti-termination depends on an intrinsic property of the phage DNA. In an effort to understand the mechanism underlying recombination, the amino acid sequences of the HK022 and  $\lambda$  integrases are being compared and sequences necessary for integrase specificity determined.

Understanding viral reverse transcriptase function has taken on a new urgency in view of the fact that HIV is a retrovirus. Using the murine leukemia virus as a paradigm, work continues to identify functionally significant sequences of the reverse transcriptase and RNase H domains of MuLV reverse transcriptase. The mechanisms underlying translational frameshifting, characteristic of retroviruses, continue to be studied by members of the retrovirus group. This year, in their search for factors controlling the suppression of a stop codon at the *gag-pol* junction, they succeeded in identifying necessary nucleotide sequences

and are at present searching for secondary structures in the mRNA that mediate viral suppression. In a related project, the structure of RNase H is being determined with the help of a newly developed high energy X-ray crystallography technique, capable of resolution to 2.2Å, developed at Columbia University.

For several years now, two groups within the LMG have been interested in how cells are able to coordinate batteries of genes in response to environmental stresses, such as starvation or amino acid deprivation. In prokaryotes the response to deprivation of an energy source or amino acids is characterized by synthesis of the nucleotide ppGpp. The nucleotide mediates a complex series of responses in *E. coli*, which enable the organism to survive. For example, whereas rRNA and tRNA synthesis are inhibited, ppGpp acts as an inducer for the operons responsible for amino acid biosynthesis. Based on their previous cloning and sequencing of the genes responsible for synthesizing (the *relA* gene) and degrading (the *spoT* gene) ppGpp, members of this group were able this year to define the functions of ppGpp in more detail. For example, by placing the *relA* gene under the control of a promoter not known to be subject to catabolite repression, they were able to confirm that ppGpp causes growth arrest. True null alleles of *relA* revealed a second route for ppGpp synthesis, which responds to glucose deprivation. By observing true null alleles of *spoT* and the effects of revertants to wild type, they were able to conclude that the likely target for ppGpp regulation is RNA polymerase. Their results also allowed them to conclude that the *spoT* gene, which bears a strong sequence homology to the *relA* gene, is bifunctional in that it is not only responsible for degrading ppGpp but also for its synthesis. The simplest eukaryote, yeast, provides a model to study how a cell responds to another form of environmental stress, namely amino acid deprivation. Amino acid deprivation causes derepression of amino acid biosynthesis, which involves a regulatory cascade. A group of researchers in the LMG has made important contributions to understanding this global control pathway by showing that the cascade is controlled at the level of translation of pre-existing mRNA for GCN4, a proximal transcriptional activator of many enzymes responsible for amino acid biosynthesis. In the past, the group identified several *trans*-acting factors that control GCN4 translation. Thus, there are at least two positive regulators needed for derepression of amino acid biosynthesis, and several more negative regulators, which repress biosynthesis under non-starvation conditions. The target of these factors has been narrowed down to four short upstream open reading frames in the 5' leader of the GCN4 transcript, which contain termination-reinitiation sequences, and without which translation of GCN4 becomes constitutive. The nature of their interaction with the transacting factors and with each other has been investigated and evidence obtained that these interactions control ribosome movement to a downstream start codon in an amino acid dependent fashion. Another important advance this year was the finding that one of the positive regulators, GCN2, is a protein kinase, and moreover, that its C-terminus bears striking sequence homology to the catalytic domain of histidyl-tRNA synthetase. This has allowed the formulation of the hypothesis that the gene encoding GCN2 is a fusion gene, i.e., it encodes a protein with two domains, one that monitors the level of uncharged tRNA in the cell, and a protein kinase domain that, under starvation conditions when the levels of uncharged tRNAs rise, becomes activated.

The frog, *Xenopus laevis*, a vertebrate whose early embryonic development is more easily studied than that of mammals, continues to provide important insights into the genetic control of early development. Progress was made this year in identifying factors important in controlling embryogenesis, especially those secreted by the *Xenopus* cell line XTC, which are active in mesoderm induction. It was established that the mesoderm induction factor (MIF), which was originally thought to be related to but is now known to be distinct from TGF- $\beta$ , induces several genes, one of which, a new homeobox containing gene called *Mix.1*, was sequenced and which appears to be involved in endoderm differentiation. Subtraction hybridization was used to enrich cDNA libraries with early genes expressed immediately after fertilization and involved in dorso-anterior axis formation. One such early gene, *DG72*, is induced in isolated ectoderm by MIF, and its sequence predicts a zinc finger and leucine zipper domain. Members of the LMG have, in the past, identified genetic markers of neural induction and, this year, have sequenced several of these genes. Others have examined the control of the expression of epidermal marker genes; in particular they have mapped with great precision the upstream regulatory region of the keratin gene in transgenic *Xenopus*

embryos, and have identified, within the region conferring tissue specificity, both a DNA binding site and the positive transcriptional regulatory factor, KTF-1, which binds to it.

The interplay between genes and cells during differentiation can be gainfully studied by investigating the hierarchical relationships of homeotic genes in *Drosophila*. Members of the LMG have worked on the influence of maternal effect homeotic genes, in particular *fsh*, and on the zygotic homeotic gene with which it most strongly interacts, *trithorax* (*trx*), at the molecular level by cloning and sequencing the gene products, and at the genetic level by observing the effects of mutations in the *fsh* gene. This year the details of processing of translation products (cleavage and glycosylation) of *fsh* were worked out, as was the sequence of the *trx* gene product; the latter contains a series of putative zinc fingers, suggesting that it is a DNA binding protein. Another group in the LMG is attempting to identify hitherto unknown genes involved in determining segment identity in *Drosophila* by their interactions with known homeotic genes, particularly the *Antennapedia* gene. Several new loci have been identified, among them *brahma* and *kismet*, which appear to exert a post-transcriptional regulatory effect on *Antennapedia*.

Several collaborative projects, involving computer simulation, NMR and spectroscopic analyses, were undertaken this year to advance our knowledge of the structure of zinc finger proteins, a large family of DNA binding proteins, and to assess the multiplicity of genes encoding zinc fingers in the human genome. In recent years, evidence has accumulated that gene expression may also be controlled at the level of RNA processing and transport. Studies have thus been initiated to investigate the role of the hnRNP proteins and have progressed to the stage of cloning and sequencing two genes encoding these proteins, as well as to the identification of their upstream promoter regions.

The newly established **Laboratory of Mammalian Genes and Development (LMGD)** is broadly concerned with the mechanisms of mammalian gene regulation, particularly during development. Members of the Laboratory are interested in identifying mammalian genes that have analogous functions to the homeotic (homeobox-containing) genes of *Drosophila*, genes responsible for controlling morphogenesis. Mouse homeobox genes so far characterized are expressed relatively late, i.e., after gastrulation, and mesoderm or neural induction. The group is therefore searching for genes that direct these early embryonic events, using degenerate homeobox oligonucleotide probes. Members of the family of *myc* genes are also known to play important roles during embryogenesis, and their expression is also being followed in order to gain insights into their function.

Another approach being pursued by this group is the creation of specific defects in genes of developmental importance, such as *c-mos* and *c-fos*, by isolating embryonic stem cells with mutations in these genes and reintroducing the cells into the mouse blastocyst. Ultimately, such chimeric mice should provide powerful tools to study a wide array of genetic functions, as well as generate mouse models of specific human diseases.

Manipulations in the transgenic mouse continue to provide important insights into the effect of stage of differentiation of a cell on neoplasia. One seminal study, in which the SV40 oncogene was directed to the lens by coupling it with the regulatory region of the mouse  $\alpha$ -A-crystallin gene, demonstrated the importance both of the timing and site of oncogene expression during differentiation for determining susceptibility of cells to transformation. Thus, the earlier in embryonic development the oncogene is expressed the more fulminant the lens tumor. It was also found that although the oncogene is expressed in all the cells of the lens, only those destined to differentiate develop tumors. These findings have important implications for the study of neoplastic growth in general. Another immortalizing oncogene, the polyoma large T antigen, coupled to the same lens promoter, is able to transform lens cells so that they proliferate *in vitro*, whereas *in vivo* the reverse is the case: cells fail to form tumors and show signs of mitotic failure. The reason for the difference between the *in vitro* and the *in vivo* response to transformation appears to be the presence, *in vivo*, of inhibitory growth factors, e.g., FGF, which are absent from the *in vitro* system.

The transgenic mouse is also a useful model to study the potential applications of transgenic animals in general. Based on their previous success with directing to the milk of the mouse human proteins of proven and potential clinical importance, such as TPA (tissue plasminogen activator) and CD4 (the HIV receptor), the Laboratory is now embarked on a

project to apply this technology to the pig and other farm animals, in collaboration with members of the USDA.

The **Laboratory of Developmental Pharmacology (LDP)** continues to elucidate various aspects of the genetic control of detoxification of environmental toxins, drugs and plant metabolites. Such detoxification generally takes place in two steps. The first is called Phase I metabolism and is catalyzed by P450 enzymes, which paradoxically results in the formation of reactive intermediates, which can be more toxic or mutagenic than their precursors, if adducts are formed with DNA. The second step is called Phase II metabolism, which converts the reactive metabolites into innocuous polar compounds with the help of conjugating enzymes. Knowledge of the control of these two Phases is important for our understanding of chemical carcinogenesis and mutagenesis in general. Moreover, genetic polymorphisms of these enzymes are thought to play a role in different susceptibilities to the deleterious effects of carcinogens. Over the past several years, the LDP has been at the forefront of efforts to penetrate the complexities of genetic regulation of detoxification. Thus, they have established that Phase I and Phase II enzymes are coordinately induced (by such environmental toxins as TCDD, also known as "agent orange") when the aryl hydrocarbon (Ah) receptor is activated. This group of genes is known as the [Ah] gene battery, and comprises the genes encoding P<sub>1</sub>450 (also known as aryl hydrocarbon hydroxylase), P<sub>3</sub>450, NAD(P):menadione reductase, glutathione transferase, aldehyde dehydrogenase and UDP glucuronosyltransferase.

Over the past few years the LDP has made major contributions to our understanding of the upstream elements controlling the constitutive and TCDD inducible expression of genes that encode Phase I metabolizing enzymes, such as P<sub>1</sub>450 and P<sub>3</sub>450. This year members of this laboratory identified *cis*-acting elements in the 5'-flanking region of the P<sub>1</sub>450 gene that are necessary (but not sufficient) for TCDD inducible gene expression. They have also now sequenced the human gene for P<sub>3</sub>450 dependent arylamine oxygenation, laying the groundwork for a deeper understanding of the polymorphism of this gene and its relationship to chemical carcinogenesis. Susceptibility to chemical carcinogenesis may also be related to polymorphism in aryl hydrocarbon hydroxylase inducibility, in particular to receptor competence. It is thus important that the structure and control of the gene for the aryl hydrocarbon receptor be elucidated. Several approaches to cloning this gene are being tested, such as transfection into Ah receptorless mouse cells of DNA that promotes TCDD inducibility, or searching for DNA-binding proteins with oligonucleotide sequence probes derived from the TCDD response element of the mouse P<sub>1</sub>450 gene.

This year efforts have also been concentrated on the genes which encode enzymes thought to play a role in protecting mammalian cells from oxidative stress and DNA damage, in particular the NAD(P):menadione oxidoreductase gene (*Nmo-1*), which encodes a Phase II enzyme that catalyzes reduction of potentially toxic oxygenated intermediates. *Nmo-1* is known to prevent, for example, the formation of highly mutagenic quinone metabolites, to decrease chromium mutagenicity by reduction, and to interfere with the covalent binding of oxygenated metabolites to microsomal proteins. Using inbred mice with a radiation induced deletion in chromosome 7, members of the LDP have identified a putative gene, which they have named *Nmo-1n*, encoding a transacting repressor for *Nmo-1* (itself located on a different chromosome). *Nmo-1* is overexpressed in these mice and, interestingly, the so-called *gadd* genes, genes that are induced by growth arrest or DNA damage (but not TCDD) are also over-expressed, suggesting that the response to this type of cellular stress may be coordinately controlled. Thus workers in the LDP may have discovered a novel negative regulatory pathway, for which *Nmo-1n* is the regulator. Unlike *Nmo-1*, the basal transcription of other members of the [Ah] gene battery, such as the Phase I genes for P<sub>1</sub>450 and P<sub>3</sub>450, is not affected by this deletion, suggesting that constitutive expression of these genes is controlled independently from TCDD induced expression. In order to understand the control of the *Nmo-1n* gene in more depth, workers at the LDP have cloned and partially sequenced it and shown that it probably corresponds to the *D1A4* locus on human chromosome 16.

The **Laboratory of Developmental Neurobiology (LDN)** continues to make important contributions to our knowledge of neuronal differentiation, and of signal transmission and reception in the nervous system.

Much effort is devoted to understanding how the developing nervous system assures the survival of certain neurones while allowing others to wither. As is known from studies in intact animals, the development of the nervous system depends on synapse selection and stabilization, but the complexity of the nervous system makes intact animals unsuitable for such studies. Therefore researchers at the LDN have developed a simple cell culture system consisting of only two cell types—spinal ventral horn cells with sensory dorsal root ganglion cells impinging on them. The system has enabled them to demonstrate *in vitro* that chronic electrical stimulation encourages the formation and efficiency of synaptic connections. By contrast, synapses not stimulated electrically weaken and lose efficiency. Communication between neuronal cells and astrocytes is critical to neuronal survival, and previous research by another group in the LDN revealed that the neurotrophic peptide, vasoactive intestinal peptide (VIP), is an important mediator of this communication. This year, efforts to delineate the role of VIP have been facilitated by the synthesis of a novel and potent antagonist to VIP. The key role of VIP during development was underscored by finding large amounts of VIP mRNA during specific periods of rapid neuronal development and differentiation. Research is now under way to identify both the targets of VIP stimulation within the astrocytes and the neurotrophic agent released from the glia in response to VIP stimulation. Neuronal cell death, one of the complications of HIV infection, continues to be another focus of the work of this group, which previously pinpointed the envelope protein (gp120) as the mediator of toxicity. Astrocytes have been tentatively identified as the target of gp120, because cell cultures predominantly composed of neurons are not vulnerable to gp120. New approaches to finding a possible treatment for neurotoxicity were opened up by the discovery that death of cultured hippocampal cells after exposure to cerebrospinal fluid from HIV patients could be prevented with peptide T, or monoclonal antibody to the putative receptor for gp120, CD4.

Intercellular neurocommunication, as is mediated by the neuropeptides derived from pro-opiomelanocortin (ACTH,  $\alpha$ -MSH and  $\beta$ -endorphin), is examined by another group in the LDN, particularly how the pro-hormones are processed and how they affect the development of the nervous system. Previous work by this group has shown the pro-hormone converting enzyme (PCE) to be calcium dependent, and this year calcium availability was pinpointed as the probable control point of this enzyme. Evidence was obtained that the qualitative differences in processing seen in different parts of the brain and pituitary may derive from differences in glycosylation of pro-opiomelanocortin. Peptides derived from POMC are known to promote cell proliferation and neurite outgrowth, and are thus likely to be of importance during embryogenesis. Therefore, an understanding of their ontogeny and the enzymatic steps that determine the availability of these peptides, should help delineate their role in development. Thus, the spatial and temporal expression of POMC mRNA was charted by determining its distribution and that of the processed peptides and their receptors as a function of embryonic age.

Mechanisms of signal transmission are investigated by a group interested in the release of neurotransmitter substance in response to the action potential train. As an experimental model, secretory vesicles are isolated from the posterior pituitary, which secrete oxytocin and vasopressin in response to depolarizing stimuli. Technical advances have enabled this group to demonstrate the importance of voltage gated calcium channels in neuropeptide release, with evidence being obtained that depolarization *per se* does not have an effect other than to open these channels. With the acquisition of a new high resolution image processing light microscope, the role of intracellular calcium distribution in the secretory process is being investigated.

Over the past years, researchers in the LDN have made important contributions to our understanding of signal reception. One group uses the pineal gland as a model for transmembrane signalling of the type that couples cyclic nucleotides to electrophysiological changes. Previously this group had found that, as in many other organs, different input signals are targeted to separate receptor mechanisms,  $\beta$ -adrenergic receptors operating through the adenyl cyclase pathway, while the  $\alpha_1$ -adrenergic receptors are coupled to the inositol phospholipid/protein kinase C pathway. The researchers established that full

activation of adenylyl and guanylyl cyclases requires both receptor types to be occupied. The problem of identifying the biochemical activation steps was approached from various directions. Thus, for example, substrates for cAMP dependent phosphorylation were identified, in particular a 33 kDa protein, similar to one in the retina, which associates with a subunit of the regulatory G protein and is unusual for being cytoplasmic rather than membrane-bound. Stimulation of potassium efflux by the  $\alpha$ -adrenergic pathway was found to be dependent on the calcium/protein kinase C mediated alkalization of the cytoplasm. Signal transduction in the pineal gland eventuates in the synthesis of melatonin (from tryptophan via serotonin). To elucidate these intracellular signal transduction steps, the genes for several enzymes responsible for the production of melatonin were cloned. The availability of these clones will allow this group to investigate how transcription of these genes is related to photoneural regulation of melatonin production.

Electrophysiological aspects of signal reception are studied by another group, which has made important contributions to our understanding of excitatory amino acid receptors, in particular glutamate receptors. One of the major achievements of these researchers was to establish that the NMDA receptor is coupled to voltage gated calcium channels. This year, using fast perfusion of hippocampal and spinal cord cell cultures under voltage clamp, they established that glycine potentiates NMDA responses by selectively inhibiting desensitization of the NMDA subtype of glutamate receptors. Kinetic analyses of NMDA receptor activity, using agonists (e.g., glutamate), modulators (e.g., glycine) and antagonists (e.g., zinc and tricyclic antidepressants), have allowed the group to build computer models to test hypotheses of receptor gating mechanisms, which should help to unravel the complex allosteric interactions involved in the regulation of receptor activity, and thus further delineate the role of the NMDA receptor in the development of neuronal circuitry, in the genesis of abnormal, seizure type electrical activity and in anoxic brain damage.

A third group seeks to understand the genetic control of signal reception during embryogenesis and development. Work is in progress to clone and sequence genes encoding ligand gated ion channels, such as those, for example, associated with glutamate receptors, and to characterize the upstream enhancer regions of the nicotinic acetyl choline receptor gene (nAChR). A 750 base pair region of the gamma subunit of the nAChR has been identified, which confers tissue specificity on the expression of these receptor genes, and is in the process of being sequenced.

The **Laboratory of Theoretical and Physical Biology (LTPB)** develops analytical and statistical methods and related computer programs to assist in various aspects of biomedical research. For example, members of the Laboratory are at the forefront of developments in the application of mass spectrometry to clinical investigations. Mass spectrometry has the general advantage of replacing potentially harmful radioactive tracers with non-radioactive isotopes to track metabolism of a variety of body constituents. This is particularly useful for the characterization of calcium metabolism in children and pregnant women. Using a multicompartmental mathematical model and the combination of two stable calcium isotopes, one administered orally and one intravenously, it is possible to characterize several important aspects of calcium metabolism. For example, bone growth in children can be measured in a matter of a few days, whereas with actual length measurements this can take several months. The measurements are based on analyses of the dilution curves of the intravenous bolus of calcium tracer, which show that the rapidly exchanging internal pool size of calcium is larger than predicted if the pool were a fixed fraction of body mass, and that differences in predicted and measured pool size are highly correlated with changes in bone mineral content, and thus bone growth. Work is in progress to confirm that excess pool size will be clinically useful as a rapid indicator of growth. This will have important implications, not only for growth but also for developing strategies to prevent the demineralization that occurs in premature infants and in osteoporosis. Improvements in thermospray liquid chromatography/mass spectrometry (ThLC/MS) developed in this Laboratory have made molecules hitherto considered too thermally labile amenable to analysis by this technique. ThLC/MS allows coupling of the mass spectrometer to high performance liquid chromatography and has been applied, for example, in a collaborative venture to measure cortisol production rates (FPR). This technique allows direct measurement of true production rates, eliminating errors derived from assumptions of plasma volume and clearance, and has

led to the surprising realization that FPR values are only half of those generally accepted, both in children and adults, with very important implications for the treatment of hypocortisolemic conditions.

Another group in the Laboratory has applied their experience in electrophoretic methods to solving problems of DNA separation by gel electrophoresis. This year, several innovations developed by the group have made it possible to resolve controversies surrounding the nature of conformational changes in operator DNA when bound to a repressor protein. One advance is the introduction of polyacrylamide, with its superior load capacity, transparency and absence of endosmosis, of a type suitable for DNA size separations in the 200 bp to 10 kbp size range. In addition, rational discontinuous buffer systems (all DNA electrophoresis is currently conducted in continuous buffers) were developed with the aid of computer programs, so as to take advantage of the sharp moving boundaries generated, which automatically concentrate the sample and provide a non-diffusing sharp reference moving boundary to facilitate measurement of electrophoretic mobilities. Electrophoretic mobility measurements allow one to characterize not only the size of molecules but also to distinguish differences in net charge, the latter by extrapolation of mobility to zero gel concentration. To test whether such extrapolation is valid, composite gels were developed to measure electrophoretic mobilities at matrix concentrations low enough to be non-sieving, but still sufficiently stable to be handled. Ascertaining physicochemical parameters, such as size and charge, requires very strict control of temperature and hydration of the electrophoretic support matrix, and substantial advances in equipment design have now made this possible; computer programs were also written with which to determine the statistical correlates of these parameters, for identity testing of electrophoretically migrating species and, in cases where separations appear difficult, to optimize separation conditions.

Other members of the Branch continue in their efforts to improve the performance of programs for the analysis of multi-ligand/receptor systems, and of bioassays and pulsatile hormone patterns, by developing new algorithms, some based on novel deconvolution techniques. Recently, much attention has been paid to the characteristics of pulsatile hormone release, which underlies the regulation of growth, sexual development and behavior. Abnormalities of pulse shape, amplitude and/or frequency may be responsible for endocrine disorders, and changes in these parameters are probably relevant to puberty and aging. Until recently, measurements of pulsatile hormone release have been hampered by lack of satisfactory theoretical underpinnings and statistical techniques for their analysis. To solve these problems, the program DETECT was developed, which can generally detect more than 90% of true peaks, while maintaining a false positive rate of 1% or less. Theoretical advances were also made for computer programs underlying radioimmunoassays, ELISA assays and bioassays, which are techniques of central importance to biomedical research. These are now based on a non-parametric algorithm, i.e., an algorithm that needs no assumptions about the shape of dose-response curves, which allows the comparison of dose-response curves that are neither linear nor logistic, even those with non-identical shapes. Other programs previously developed by members of the Laboratory to facilitate the analysis of complex multiligand binding experiments, such as DESIGN and LIGAND, were used, for example, to establish that the neurotoxic effects of homocysteic acid, the amino acid accumulating in excess in those suffering from the genetic disease homocystinuria, are most likely due to its affinity for NMDA receptors, as are the effects of PCP, which appears to bind to a site allosterically coupled to the NMDA receptor.

Another aspect of the Laboratory's work is the application of their statistical and programming expertise to the management of diabetes. This year, based on pharmacokinetics and mathematical modelling, the program GLUCOJECT was developed to allow predictions about blood glucose levels in response to variations in diet and insulin regimen.

The **Human Genetics Branch (HGB)** is responsible for studying the etiology, diagnosis and treatment of children with genetic diseases. The Branch also has an interest in various aspects of mammalian development and cellular differentiation. In particular, members of the Branch direct their efforts to understanding and treating lysosomal transport defects, such as cystinosis and sialic acid storage diseases, and defects in connective tissue formation, such as osteogenesis imperfecta. Other genetic diseases receiving their attention are cystic fibrosis and disorders due to defects in drug-conjugating enzyme systems.

The HGB investigates a large number of patients with cystinosis. Ongoing clinical trials of cysteamine treatment for this disease are showing great promise, particularly in delaying and attenuating the renal and corneal complications of this disease. (Cysteamine permits solubilization and lysosomal transport of cystine, which otherwise crystallizes in and engorges the lysosomes. Cystinotic lysosomes have been shown by this group to be defective in a putative integral membrane transport protein.) Studies are also being conducted to test whether carnitine supplements to the diets of cystinotic patients, whose kidneys are no longer able to reabsorb this amino acid, may prevent the myopathies associated with renal tubular failure (Fanconi syndrome). Studies on rare metabolic diseases often yield unexpected insights into normal physiological processes, as was the case this year with patients suffering from sialic acid disorders. Thus it was found that sialic acid is filtered by the kidney but not reabsorbed. As part of the Branch's program to study sialic acid storage diseases, which are usually due to defects in transport of the charged sugar across the lysosomal membrane, an extremely rare form of sialuria was discovered, in which sialic acid is stored in the cytosol rather than within the lysosome. In patients with sialuria, excess production of sialic acid is due to failure of the end-product in the biosynthetic pathway to cause allosteric inhibition of the epimerase that controls the pathway; this may well be the first time that an allosteric defect, as contrasted with a catalytic defect, has been found to underlie an inherited metabolic disease. As part of their interest in mechanisms of lysosomal transport, HGB investigators are also studying the moniodotyrosine carrier system, which they have shown to be responsive to thyrotropin (TSH). This system is critical to thyroid hormone production, and attempts are being made to clone the gene for the carrier protein.

Other members of the Branch concentrate their research efforts on patients with osteogenesis imperfecta (OI), commonly known as "brittle bone disease", and on understanding the molecular nature of this disease, the most common heritable disorder of connective tissue. Over the last few years the group has made substantial contributions to understanding the etiology of OI, using new techniques of molecular biology to locate point mutations in the genes for type I collagen, which is where the defect is thought to lie. OI appears to be caused by single amino acid substitutions, which perturb helix formation and lead, in turn, to over-hydroxylation or over-glycosylation. Hitherto, regions containing point mutations have been located by conventional protein sequencing techniques, which require pure protein preparations, are relatively insensitive, and which are not able to distinguish between the two chains of the helix. The collagen genes are difficult to sequence by the usual hybridization mismatch techniques because of their large size and because their repetitive structure favors internal hybridization. Members of the group have overcome these problems by synthesizing a complete series of overlapping antisense riboprobes from the cDNA of normal procollagen genes and by using these probes to detect mismatches in hybrids formed with cDNA from cultured fibroblasts of OI patients. Subsequently, regions containing the mismatches are amplified by the polymerase chain reaction. The technique has enabled the group to locate point mutations in two types of OI, perinatal lethal type II, and type IV, which results in a milder form of the disease. The nucleic acid sequence data agree with those obtained by conventional protein sequencing, and show both OI subclasses to be the result of single base substitutions, both causing the replacement of glycine with more bulky helix-perturbing amino acids. These data have advanced our understanding of the critical structural requirements for a healthy bone matrix, knowledge that will deepen as more mutations are mapped and their locations related to the severity of the disease. The techniques developed by the group will facilitate prenatal diagnosis, as well as studies of other connective tissue disorders.

Another group of workers in the HGB is directing its efforts to understanding the mechanism whereby certain steroids inhibit inflammatory responses of the mucosal epithelia, in particular the role of uteroglobin, which is induced by glucocorticoids. Uteroglobin is of interest because it is produced in large amounts by the pregnant rabbit uterus, where its role may be to inhibit responses that would otherwise lead to fetal rejection. A notable success this year was the production in *E. coli* of recombinant uteroglobin by a novel vector. This vector permits efficient bacterial expression of a eukaryotic gene encoding a protein that contains two disulfide bridges in its quaternary structure. Uteroglobin inhibits phospholipase A<sub>2</sub> (PLA<sub>2</sub>), a key enzyme controlling the arachidonic cascade. Site directed mutagenesis studies are now being conducted in this bacterial expression system to determine the

important functional sequences of uteroglobin. In a collaborative study, tissue specific expression of cloned rabbit uteroglobin DNA was achieved in transgenic mice, which should provide a useful model for the further study of the function of this protein. Progress was also made in predicting the structure of a general class of non-steroidal antiinflammatory agents, capable of inhibiting the synthesis of all eicosanoids, by identifying sequences of uteroglobin and another phospholipase inhibitor, lipocortin I, that demonstrate potent anti PLA<sub>2</sub> activity. Work has also been done on the mechanism of activation of PLA<sub>2</sub>, which is an important element of many cellular signal transduction systems. The findings show a novel activation mechanism by transglutaminase.

Members of the HGB are interested in the mechanisms of differentiation of the liver and, in cell lines derived from fetal hepatocytes, are studying how the expression of certain liver specific proteins is developmentally regulated. They have succeeded in cloning both the fetal and adult cDNA of one such protein, alpha-fetoprotein, and have made progress in comparing their nucleotide sequences to determine how this gene is controlled. They are also investigating the role of glucocorticoid hormones and retinoic acid in differentiation by examining the induction of other liver specific genes, such as tyrosine aminotransferase and albumin, in a reversible differentiated-dedifferentiated hepatocyte cell line, which they produced by transformation with a temperature sensitive mutant of SV40, and which is proving to be an excellent model for such studies.

Two projects in progress in the Branch relate to pregnancy-associated disorders. One project involves studies of a protein that is a potential marker for choriocarcinoma, a human pregnancy specific  $\beta_1$ -glycoprotein (PS- $\beta$ -G), which is also elevated in the sera of most patients with hydatiform mole and invasive mole. The group has characterized various forms of PS- $\beta$ -G in an attempt to determine the function of this protein. It shows strong sequence homology to human carcinoembryonic antigen, and may indeed be its normal placental counterpart. The other project concerns one of the major preventable causes of mental retardation, fetal alcohol syndrome (FAS). HGB investigators hypothesize that maternal alcohol consumption may be particularly damaging to fetuses that have an aberrant gene for transketolase, a thiamine dependent enzyme, because alcoholism results in thiamine deficiency. Preliminary data show that fibroblasts cultured from FAS patients have a higher K<sub>m</sub> for thiamine pyrophosphate compared with controls. A newly available cDNA clone for the human liver transketolase gene should help to determine whether infants with FAS do have a specific abnormality in the transketolase enzyme gene.

To pinpoint the genetic lesion in bilirubin transferase of patients suffering from Crigler-Najjar and Gilbert's syndrome, a unit within the Branch has been characterizing by molecular cloning a wide range of UDP glucuronosyl transferases (UDPGT), which are often functionally coupled by coordinate control with Phase I P450 monooxygenases. This year has brought the isolation and partial sequencing of two human clones of UDPGT, one of which is a novel estrogen conjugating enzyme. These will be used to determine allelic differences between the genes of the defective transferase and their normal counterparts in an attempt to develop a specific diagnostic tool for these patients.

**The Cell Biology and Metabolism Branch (CBMB)** continues in its position of preeminence in several areas of research, notably the genetic control of iron metabolism, the structures of the T-cell antigen and interleukin-2 receptors, and the cell biology of secretory processes.

Spurred by the desire to understand the mechanism of hemochromatosis, a common genetic disorder resulting from an inability to control cellular iron levels, members of the CBMB discovered a few years ago that cellular iron levels are mainly controlled at the translational level. The messenger RNAs for both the transferrin receptor (TfR) and ferritin, the proteins responsible for, respectively, transporting iron into, and sequestering excess iron within the cell, contain a unique nucleotide sequence, called the iron responsive element (IRE). Recently these investigators discovered a binding protein with affinity for the IRE (IRE-BP); the protein prevents translation when bound to ferritin mRNA and degradation when bound to the TfR mRNA. Iron changes the affinity of the IRE-BP for the IRE, a low affinity state predominating when iron is abundant and a high affinity state when iron is scarce. This explains how excess iron can promote the synthesis of ferritin and have the opposite effect on iron uptake. This year, a group of CBMB workers developed a novel binding assay, based on gel mobility shift quantitation, which allowed them to show that

affinity states depend on the oxidation state of sulfhydryl groups in the IRE-BP, which are changed by fluctuations in the concentration of available iron. The idea that "sulfhydryl switches", depending on their individual oxidation potentials, effect allosteric changes in proteins is of great importance to cellular protein function in general. With their new RNA affinity purification technique, the group was able to isolate quantities sufficient to sequence large portions of the IRE-BP, and, as a result, to start efforts to clone the gene encoding the IRE-BP.

This year also brought some important insights into the cellular mechanisms and genetic control of iron uptake. Using the simplest eukaryote to facilitate genetic analysis, members of the CBMB have been able to produce mutants of yeast incapable of taking up iron. With these they were able to demonstrate that the defect resides in the gene encoding an externally oriented iron reductase, which is highly regulated by the iron status of the cell. This is the first time that ferric reductase has been identified as part of the iron uptake mechanism for any organism. They have been able to clone this gene, which will enable them to study the control of iron uptake in more detail.

In another area of research, the CBMB has, over the past few years, spearheaded the characterization of two important receptors of the immune system, the interleukin-2 receptor (IL2R) and the T cell antigen receptor (TCR). Two years ago members of the CBMB discovered that, in addition to the known alpha chain, the IL2R contains a second subunit, the beta chain; this year they demonstrated that the three known affinity classes of the receptor for IL-2 reflect different combinations of the two subunit chains. The signalling functions of two components of the TCR complex, the zeta chain and its homolog, the eta chain (the latter discovered by this group a year ago), continue to be elucidated by the group. Using mutant variants of T cells lacking eta, the group has shown recently that both subunits are necessary to couple the receptor to the phosphatidyl inositol activation pathway; in its absence only the tyrosine kinase pathway is activated. However, T cells without eta produce normal amounts of IL-2, raising the question of whether its production is stimulated via the tyrosine kinase pathway. This question was answered in an elegant set of experiments using T cell hybridoma cells stably transfected with the *v-src* oncogene, which encodes a tyrosine kinase. Functionally, expression of this gene was able to mimic the results of receptor-mediated kinase activation, such as IL-2 production. On a biochemical level, tyrosine kinase phosphorylated substrates were identified, the most notable of which is the zeta chain of the TCR. Examination of the phosphorylated substrates led to the discovery of other receptor activated tyrosine phosphorylation pathways, e.g., one that is linked to the membrane phosphatase, CD45, and another linked to the *c-raf* serine threonine kinase, which itself is phosphorylated via the protein kinase C pathway.

In addition to studying the structure and function of the TCR, the CBMB continues to make seminal contributions to the cell biology of its assembly and cell surface expression, and to apply the insights gained to the role of the endoplasmic reticulum (ER) in the formation of multicomponent complexes in general and to its role in the secretory system. In the past, CBMB workers have observed that different subunits of the TCR vary in their susceptibility to degradation in the ER, depending on their state of assembly. Gene splicing and transfection experiments have allowed them to pinpoint the transmembrane sequence of the alpha subunit as a signal for degradation by the ER. If this sequence is attached to a protein that is resistant to ER degradation, such as the IL-2 receptor, the latter acquires susceptibility to degradation.

In a related project use was made of a new blocking agent, Brefeldin A, which prevents the movement of membranes from the ER to the Golgi apparatus. CBMB researchers showed that the Golgi is recycled into the ER, challenging the current dogma of secretion as a one way street. Evidence was presented that this retrograde movement of Golgi vesicles depends on microtubules. Brefeldin A also allowed them to demonstrate the importance of the ER traffic pattern for the MHC class I associated presentation of endogenous antigens.

The **Laboratory of Developmental and Molecular Immunity (LDMI)** continues its innovative program of vaccine development, which is largely based on the premise that T-dependent immunogenicity can be conferred on surface carbohydrate antigens by conjugating them with carrier proteins, such as tetanus toxoid (TT), which in themselves are useful antigens. This approach, which was originally developed by researchers at the LDMI, has proven very

fruitful, culminating this year in the licensing of a conjugate vaccine against *Haemophilus influenzae* type b (Hib) meningitis, the leading cause of acquired mental retardation in the USA, to protect children 18 months and older. The new vaccine overcomes the problems of vaccine failures associated with the currently used, unconjugated polysaccharide vaccine, which does not protect infants, the age group at greatest risk for Hib infection. Clinical trials are now near completion that demonstrate the effectiveness of this vaccine also in infants, making licensure for immunization of infants likely within the next year or two.

Several other projects are underway to apply the conjugation technology to surface components of several pathogens, against which no effective vaccines have been produced by conventional techniques. A conjugate of pneumococcus type 6 surface polysaccharide with TT was successfully tested in children at high risk for developing pneumonia and sepsis, such as those suffering from sickle cell anemia. The experimental vaccine elicits protective antibody levels, whereas the polysaccharide alone is ineffective. Several other conjugate vaccines have been shown to be effective in animal tests. Thus for example, conjugates of surface polysaccharide of pneumococcus type 14, the most common cause of otitis media, with pertussis toxin are effective in eliciting protective antibody levels against both antigens, as is TT conjugated with the capsular polysaccharide of group B streptococcus type III, the most common cause of neonatal sepsis.

Of potentially wide significance to world health is the development of conjugate vaccines against enteric fevers, which continue to cause high morbidity and mortality in the developing nations. A few years ago the LDMI produced a highly successful capsular polysaccharide vaccine against *Salmonella typhimurium*, which is far superior to the currently used cellular vaccine, as determined in a 2 year study in a high risk population in Nepal. This vaccine protects 70% of the recipients for about two years, whereafter protection rates fall. To improve its immunogenicity and prolong protection, the LDMI has therefore produced a TT conjugate version of this vaccine, which is now being tested in adult volunteers. In an attempt to confer simultaneous protection against two enteric fevers, techniques are being developed to conjugate surface lipopolysaccharides of *Shigella* or various non-typhoidal *Salmonellae* to such antigens as the  $\beta$ -subunit of cholera toxin.

Research conducted over the past few years by members of the LDMI has paved the way for replacement of the controversial cellular vaccine against pertussis with one that consists only of toxoided pertussis toxin, which should yield a more effective vaccine and eliminate the worrisome neurological side effects, which occasionally occur with whole cell vaccines against this pathogen. Optimal dosages of this new vaccine have been established and clinical trials have progressed to the final stages of assessing its effectiveness when compounded with diphtheria and tetanus toxoid.

The other major thrust of the work of the LDMI is to address the mechanisms of gene regulation in the developing immune system, in particular how transcription is controlled of genes that are critical to T cell recognition of foreign antigens. The body's capacity to recognize antigens is under the direction of the major histocompatibility antigen complex (MHC) class I genes. Understanding how their expression is controlled thus has enormously important implications for transplantation biology, recognition and elimination of pathogens, maternal-fetal interactions, immune diseases and possibly cancer. Over the past few years, this group of researchers has succeeded in characterizing the 5' upstream regulatory region (enhancer/promoter) of these genes. They have shown that the regulatory region contains several DNA elements, one of which confers interferon responsiveness on the expression of MHC class I genes, others which control the development of neonatal expression, and still others that repress transcription. This year much progress was made in identifying and sequencing several of the protein factors that bind to various domains of the 5' upstream regulatory region and thus control their activity. The amino acid sequence of one of these, the H-2RII binding protein, revealed that the protein contains a putative zinc finger with strong sequence homology to several hormone receptors, and that it even binds to one of the hormone response elements, the estrogen response element of the vitellogenin gene, suggesting that this factor may be capable of regulating not only MHC class I genes, but also other genes controlled by similar DNA sequences.

Another nuclear protein factor to be identified and partially sequenced by this group is one that, upon binding to part of the regulatory region, confers interferon responsiveness on MHC class I gene expression. This group of workers has shown in the past that MHC class

I expression is repressed during embryogenesis, expression beginning during the neonatal period. This year the regulatory region was established as the site of this repression and a protein factor identified that, by binding to a regulatory sequence in the region, causes the repression.

The overall objectives of the **Developmental Endocrinology Branch (DEB)** are to deepen our understanding of endocrine disorders affecting reproduction and growth, and, based upon their research results, to develop more precise diagnostic tools and to improve treatment.

In the past year, several groups of workers within the Branch have made much progress in studies on fertility. Protocols to assess the fertility of healthy couples have revealed an unexpectedly high incidence (21%) of spontaneous abortions during the first month of pregnancy. These early pregnancy losses, which can only be recognised by an increase in urinary hCG, appear to be a normal occurrence, in that they are associated with a relatively high subsequent rate of conception. As a consequence, a recommendation has been formulated that women suffering early spontaneous abortion not be advised to postpone pregnancy, as is now the practice. In related studies on the functions of hCG carbohydrate structure, it was found that although desialylated and deglycosylated hCG are inhibitors of adenyl cyclase activity, they stimulate testosterone secretion *in vivo* to the same extent as native hCG, probably because of the large excess of receptors for hCG present in the Leydig cell. Another interesting carbohydrate variant of hCG is one that bears galactose at the  $\beta$ -carboxy terminal. It is secreted in large amounts by patients with gestational trophoblastic neoplasia, and is thus a potentially important marker for this disease. Much infertility is attributed to luteal phase dysfunction without a clear understanding of its etiology, or a calibrated diagnostic tool. The feasibility of using a new non-invasive diagnostic method for detecting luteal dysfunction was tested (the currently available method relies on endometrial biopsy), which is based on the absence of a progesterone response to an hCG challenge; preliminary results confirm the utility of this approach. Lactational amenorrhea is the most common method to space children in many parts of the world, yet its underlying physiology is poorly understood. Members of the Branch determined that it has a neuroendocrine basis by observing depression of gonadotrophin release during suckling.

In another area, DEB workers continue to refine the differential diagnosis of delayed puberty. Experiments were initiated to explore the feasibility of using the NMDA receptor pathway, which is known to trigger the pulsatile release of LHRH in rhesus monkeys, to test the integrity of the gonadotroph—an approach which shows great promise. The DEB is also at the forefront of new developments in the treatment of familial male precocious puberty and secondary central precocious puberty. Therapy is aimed at inhibiting the accelerated growth and premature epiphyseal fusion resulting from overproduction of androgens and their derivatives, estrogens, using a combination of an antiandrogen and an aromatase inhibitor. In the case of secondary central precocious puberty, the efficacy of adding LHRH analog therapy to this treatment regimen is under investigation. Interim data on normalization of growth rates are very encouraging. These are long term clinical trials involving children whose ultimate reproductive competence will also be of great interest.

Research on linear growth is a second major focus of the Branch. Improvement in the treatment of growth impairment is based on the recognition that most cases of apparent growth hormone (GH) deficiency result from lack of GH releasing hormone (GHRH), rather than GH *per se*. Clinical studies have shown that the usual intensive regimen of several GH injections per day can be replaced with a single dose of GHRH to restore normal growth, and that a suppressor of somatostatin (the "anti-growth" factor) can be used to improve the response to GHRH. Another approach of promise is based on the finding that a hexapeptide derived from enkephalin has a potent effect on the release of GH, and its potential for oral administration as well as its mode of action are being investigated. Novel approaches to the non-surgical treatment of growth hormone-secreting pituitary tumors that are resistant to other curative measures are being developed, such as linking GHRH to toxins or neutron beam-sensitive organo-boranes. Success in this type of approach will have important ramifications for the treatment of hormone-dependent tumors in general. Members of the Branch also undertook a reevaluation of methods to test GH deficiency, finding the assay for spontaneous GH release to be inferior to GH stimulation tests. Standardization of the GH stimulation test is now in progress, as is the development of normative standards for this test

with regard to age, sex and pubertal age. The development of such standards is also the *sine qua non* for the treatment of childhood disorders of the pituitary-thyroid axis. Members of the DEB have therefore established age-related blood levels of thyroid hormones, thyroxine binding globulin (TBG) and TSH, and have found that children display a similar nocturnal surge in TSH secretion to adults. Importantly, the nocturnal TSH surge was found to be blunted in children with borderline central hypothyroidism, and this characteristic appears to be the most sensitive test yet described for this disorder. The same group of workers found that thyroid hormone levels and TBG both decline with age, leading to the hypothesis that developmental changes in thyroid hormone levels are due to alterations in thyroid hormone transport.

Members of the DEB continue to make important contributions to our understanding of the hypothalamic/pituitary/adrenal axis (HPA). The use of corticotropin releasing hormone (CRH) has continued to revolutionize the diagnosis of a variety of disorders of the HPA axis, namely Cushing's syndrome, adrenal insufficiency and hypercortisolemic affective disorders. Thus CRH administration causes elevations in plasma ACTH and cortisol in patients with Cushing's disease (due to pituitary adenomas) but not in patients with ectopic ACTH syndrome, providing a simple outpatient diagnostic test. CRH can also be used to distinguish between adrenal insufficiency due to hypothalamic and pituitary causes because, in the former case, there is an ACTH response, whereas in the latter there is not. Similarly hypercortisolemic psychiatric disorders can be distinguished from true Cushing's disease because the ACTH response to CRH is blunted in the former condition. In a separate study, standards were established under which CAT and MRI scans can be used as adjunctive diagnostic tools to help distinguish Cushing's syndrome from ectopic ACTH secreting neoplasms or from micronodular adrenal disease, which is critical to the appropriate treatment of these conditions. The regulation of CRH secretion was investigated in hypothalamic organ cultures by studying the effects of various secretagogues. Of particular significance is the finding that several cytokines are potent secretagogues, strongly suggesting that the CRH neuron of the hypothalamus is the link between the immune system and the CNS. This link was further demonstrated in a collaborative effort with two other Institutes; the competitive inhibitor of cortisol binding, RU 486, was used to confirm the importance of the HPA axis, in particular the CRH neuron, in preventing arthritic responses to immunogens. Thus, the Lewis strain of rats, which tends to develop arthritic lesions after challenge with an immunogen, such as streptococcal cell wall polysaccharide, was shown to have a defective CRH response to stressors; histocompatible, and thus closely related rats, which do not normally develop arthritis after receiving the same immunogen, did so if also administered RU 486.

**The Laboratory of Comparative Ethology (LCE)** engages in research designed to quantitate with sophisticated statistical techniques the genetic, environmental and physiological determinants of behavior, particularly as these relate to the impact of parenting on human development and to language development. These questions are approached either by studying non-human primates, experimental paradigms amenable to selective breeding and other manipulations, or by observing large samples of human infants interacting with their care-givers.

Genetic determinants of temperament and social behavior are examined in rhesus monkeys, animals which resemble humans in many aspects of their behavioral development. Members of the LCE have, in the past, established that certain personality traits, such as "laid-back" (phlegmatic) and "uptight" (anxious and fearful, also called reactive) temperaments, will breed true through successive generations and are correlated with the reactivity of the hypothalamic-pituitary axis and vagal tone. Research on the effect of the environment on monkeys with these two types of genetically determined temperament has yielded some provocative findings this year. For example, cross-fostering showed that nurturing mothers can promote apparently normal behavioral development in reactive rhesus monkeys, and that such infants later perform better on some tests of cognition than "laid-back" monkeys, and, in fact, later rise to the top of the social dominance hierarchy. However, reactive monkeys reared by punitive mothers stay at the bottom, demonstrating that rearing styles are of particular importance for reactive personalities. Conversely, parenting styles among these monkeys have no effect on the poor coping behavior and passivity of

reactive infants when they are subjected to the stress of brief separation from their caregiver. On the other hand, there is a marked effect of parenting style on the coping behavior of "laid-back" infants; when they are raised by punitive foster-mothers they demonstrate coping skills in response to stress that are as poor as those of the reactive infants. Clearly, the optimum combination is a "laid-back" infant with a nurturant mother, although the "uptight" genotype must have some survival advantage. "Laid-back" and "uptight" genotypes are also evident in the wild, as has been observed in rhesus monkey colonies in Cayo Santiago, PR. Monkeys of the "laid-back" type are more likely to emigrate successfully from their natal troop to an adult peer group than the uptight monkeys. Temperament and its physiological concomitants (vagal tone and plasma cortisol levels) are the only parameters so far found to correlate with emigration behavior. Interestingly, temperament has also been shown to correlate with vagal tone in human infants, as work by other groups in the LCE has demonstrated.

In other studies of rhesus monkeys, additional physiological corollaries of behavior have emerged, such as serotonin metabolite levels in the CSF, which parallel timidity and despair-like behavior, and sex-specific changes in adreno-cortical response to challenge during adolescence. Thus, cortisol response to stress declines in prepubescent male monkeys, whereas in prepubescent females it either remains stable or increases. These differences precede puberty by as much as a year, and in males, may have adaptive advantages.

In a collaborative study with NIAAA, genetic and environmental factors predisposing to drinking behavior were assessed, again using rhesus monkeys bred for different temperaments. In general, monkeys raised by their mothers are much less likely to drink alcohol when offered than monkeys raised with their peers, but separation stress obliterates the effect of rearing practices, causing monkeys with reactive temperaments to drink much more than their non-reactive peers, whether they were raised by their mothers or with peers.

Another group of LCE investigators interested in the neurophysiological basis of language formation have located in the brain biochemical components of the monoamine oxidase A pathway and regions of the limbic system important for the production of distress calls in infant squirrel monkeys. Social prerequisites of vocalization in infant monkeys are being established, a notable finding this year being that, until an infant leaves its mother's back, aunts engage in much more vocal exchange with infants than do their mothers.

Several projects are devoted to the determinants of human behavior, the other major emphasis of the LCE's research. The current interest in the long term consequences of early day-care experience is reflected in the amount of research devoted to this question. Thus, statistical evidence was presented that the type of day-care is less important to social and intellectual development than is the quality, and also that instability of early day-care arrangements is particularly detrimental. Child rearing practices often differ more fundamentally among cultures than within a single culture, which is why the LCE has several projects in progress to examine the effects of these intercultural variations on childhood development. The data collection is largely complete and analysis is in progress.

A long term goal of one group of workers within the LCE is to determine which "intrinsic" (presumably genetic) attributes of infants and children are predictive of future cognitive and social development, and to measure how these are affected by complex, inter-related parental behaviors. In the past, the group established that habituation efficiency in infants (the rate at which infants become bored with a presented stimulus), an indicator of "intrinsic" attributes, predicts later cognitive abilities. This year the group has turned their attention to the influence of parental activity on different aspects of cognitive competence in toddlers, particularly how different domains of parental activity are interrelated and inter-dependent. So far, studies on infant and toddler development have not taken these conditional relationships into account. Members of the LCE are now attempting to do this with the help of structural equation modeling, which allows the experimenter to assess statistically the effects of combined variables. Various infant and toddler skills are examined, such as language development and play competence. Preliminary results are demonstrating the advantages of this approach. For example, measured independently, neither maternal didactic behavior towards infants nor the social interactions within the mother-child dyad have any effect on pretend play, whereas the combination of the two maternal behaviors does have a significant effect. This statistical approach has also allowed LCE researchers to distinguish the effects of maternal responsiveness, maternal didactic

encouragement, and infants' habitational competence and to establish that, to some extent, infants who are more efficient at habituation encourage their mother's responsiveness. The most striking finding to emerge this year is that at 5 months maternal contingent responsiveness, i.e., responsiveness to the infant's activities (other than crying), predicts exploratory (attention-play) and representational (play-language) competence at 13 and 20 months.

The **Endocrinology and Reproduction Research Branch (ERRB)** conducts research into mechanisms of hormone secretion and hormonal control of cellular metabolism in a variety of target organs, with particular emphasis on the nature of various signal transduction pathways. The Branch is also concerned with the relationship of the structure of peptide hormones to their function.

Pituitary secretion of peptide hormones continues to be studied in several projects. For example, members of the Branch have shown that luteinizing hormone (LH) release from the pituitary is under the control of not only gonadotropin releasing hormone (GnRH) but also the neurohypophyseal hormone, oxytocin, which acts via a different receptor to stimulate LH release, probably affecting baseline secretion, whereas GnRH elicits pulsatile LH release. Previous studies by this group have demonstrated the importance of bioassay, as opposed to immunoassay, of LH, particularly in clinical studies. Bioassay has allowed the group to show that low dose pulses of GnRH cause pulsatile changes in serum LH in humans, which could be due either to increases in LH release or clearance. Using computer modelling it has been possible to distinguish between these two possibilities and define the kinetic parameters of normal fluctuations in serum LH levels, giving reasonably accurate measures of the half life of bioactive serum LH without having to resort to radioactively labelled hormone. Bioassay has also been used to establish the role of estrogens in regulating GnRH stimulation of LH secretion. In postmenopausal women on estrogen replacement therapy, it was found that estrogen enhances the responsiveness of the pituitary to GnRH stimulation, which helps explain the pre-ovulatory LH surge.

The first step in hormonal activation of cellular processes is binding of a hormone to a target cell, and the Branch has been at the forefront of efforts to purify peptide hormone receptors, in order to study their functions. This year, cell surface receptors for GnRH and corticotropin releasing hormone (CRF) were solubilized in active form and their molecular weights determined. Like other cell surface receptors, the CRF receptor appears to be heavily glycosylated. The previously purified LH receptor protein was sequenced this year. Novel methods are being designed to characterize the unstable angiotensin II (AII) receptor (recently proposed to correspond to the *mas* oncogene product) by cloning the gene. One approach members of the Branch have developed is to screen adrenal mRNA in *Xenopus* oocytes by monitoring for AII-sensitive  $\text{Ca}^{2+}$  release, using the  $\text{Ca}^{2+}$  sensitive light emitter, aequorin. Advances in the area of receptor activation include the demonstration that LH and hCG control receptor phosphorylation by changing the conformation of the receptor.

Conformational changes in cell surface receptors are linked to different membrane transduction pathways, and the Branch has continued to define which of these is used by each of the different peptide hormones. Thus, it was found that voltage dependent calcium channels are involved in the sustained phase of LH release from gonadotrophs, whereas the initial, rapid release depends on intracellular calcium mobilization. A novel G protein-dependent signal transduction system, not linked to the cAMP pathway, was discovered in prolactin dependent lymphoma cells. Autocrine CRF secreted by the testis inhibits the effects of LH but appears to act through a different transduction pathway than LH, inhibiting protein kinase C (PKC), which is part of the phosphatidyl inositol pathway (PI), whereas LH itself acts through cAMP. AII activates aldosterone production via the PI transduction pathway, but the role of PKC in translating AII action into stimulation of steroidogenesis in the adrenal glomerulosa is controversial. Experiments have shown that, although AII promotes the translocation of PKC, which is presumed to precede its activation, this is not responsible for the stimulation of steroidogenesis, but rather appears to promote cell growth.

Another group of researchers within the ERRB continues to uncover aspects of the metabolism of inositol polyphosphates (IP), as they relate to the action of AII. Evidence was obtained that AII promotes the turnover of IP by two separate pathways, the hydrolysis of

inositol triphosphate to inositol 1,4-bisphosphate and inositol 4-monophosphate, and a phosphorylation-dephosphorylation cycle involving tetrakis- and pentakis-phosphates of inositol, and that the latter are involved in maintaining elevations in intracellular calcium levels, necessary for the sustained phase of aldosterone secretion.

PKC continues to receive the attention of several members of the Branch. In the past, they have shown that PKC is crucial to many cell regulatory processes, in addition to those regulated by hormones. PKC requires  $\text{Ca}^{2+}$  and phospholipids for activation, and the characteristics of this interaction were examined; it was found that  $\text{Ca}^{2+}$  binds directly to the enzyme and that subsequent binding of phospholipids leads to its activation; the reverse order of binding leads to inactivation. Two years ago members of the Branch discovered that PKC exists in three major isozyme forms and, in order to determine their physiological roles, biochemical methods are being sought to distinguish the isozymes. It has been shown, for example, that they display different susceptibilities to phospholipid inactivation. Magainins, natural amphiphilic peptides with antimicrobial properties, can be phosphorylated by PKC, suggesting that some of their actions may be mediated by PKC. Some analogues of magainins, synthesized by members of the Branch, show selective inhibition of the different isozymes and may also be useful agents for distinguishing them. Changes in the distribution of PKC isozymes in the cerebellum of the post-natal rat confirm that the three isozymes are under separate genetic control and that their distribution is cell specific, type I being found in the Purkinje cell bodies and dendrites, type II and III in the granule cells (but with non-identical distributions), and type III in the cell bodies, but not dendrites, of the Purkinje cells. Studies on glutamate sensitive granule cells from the cerebellum show different susceptibilities to down-regulation by a phorbol ester (phorbol esters are synthetic activators of PKC) and different degrees of translocation from cytosol to membrane fractions in response to glutamate stimulation. Investigation of PKC inhibitors is useful also from another standpoint, because some of these may have therapeutic potential. One such inhibitor, H-7, can prevent CD4 phosphorylation and thus infection of cultured T-cells by HIV. Suramin, an inhibitor of HIV reverse transcriptase, was tested for its action on PKC, and found to be selective in its inhibition of the various isozymes, PKC I being most sensitive. PKC is thought to be activated proteolytically (the activated enzyme being called PKM) and this activation was investigated using myosin light chain as a substrate. PKC itself only phosphorylates at three sites, whereas PKM phosphorylates both at these sites and at two additional sites, those normally phosphorylated by myosin kinase. This is the first time that changes in substrate specificity have been demonstrated following proteolytic activation of PKC. The structure of PKC has been investigated using deletion analysis of PKC cDNA expressed in COS cells. This allowed identification of regions of the molecule important for various functions. For example, a cysteine rich zinc-finger region was identified, which appears to be necessary for phorbol ester binding.

Other hormonal systems receiving the attention of the Branch are the autocrine control of differentiation of ovarian granulosa cells by IGF-1 (insulin-like growth factor 1); paracrine control of progesterone production by ovarian growth hormone and AII; the role of AII in fetal development; the role of steroid binding proteins in adrenal steroidogenesis; and the mechanism of desensitization of steroidogenesis in the testis as a consequence of down-regulation of LH receptors. This down-regulation appears to occur at the transcriptional level, by controlling the level of mRNA for the enzyme responsible for converting  $\text{C}_{21}$ -steroids to the  $\text{C}_{17}$ -androgens. The gene for this enzyme, 17- $\alpha$ -hydroxylase/17-20-desmolase, has been cloned and structural features of the enzyme analyzed from the deduced amino acid sequence.

Another group in the ERRB is concerned with the molecular structure of biologically active peptides and peptide hormones. This year, for example, the group worked on the design of antimicrobial agents based on the magainins. These peptides have the disadvantage of being hemolytic, which limits their potential clinical usefulness, and the group has been involved in efforts to increase the ratio of antimicrobial to hemolytic properties by constructing analogues. They have succeeded in doing this by increasing the  $\alpha$ -helicity of the molecules by substituting those amino acids with a low propensity to form  $\alpha$ -helices with L-alanine, which has the opposite propensity. This has increased antimicrobial activity *in vitro* by two orders of magnitude, without any appreciable effect on hemolysis. The

magainin analog with the highest degree of  $\alpha$ -helicity binds best to synthetic lipid membranes, which is the probable basis for its action.

In the **Laboratory of the Scientific Director**, the focus is on illuminating the molecular mechanisms of DNA replication, mutagenesis and repair, using *in vitro* and *in vivo* eukaryotic and prokaryotic model systems. This work is fundamental to the study of genetic disorders, carcinogenesis, and birth defects. Work by this laboratory on mutation fixation has been greatly aided by the use of DNA virus-based shuttle vectors to investigate the molecular mechanisms by which agents that damage DNA induce mutations in mammalian cells. The pZ189 vector, based on the SV40 virus, has proven to be very useful in studying the direct and indirect effects of two typical DNA damaging agents, UV and a derivative of the carcinogen, benzpyrene. The vector can be treated with mutagens (*in vitro* or *in vivo*); it is replicated in primate cells, harvested, and then amplified in bacteria to produce sufficient material to analyze the mutations that had occurred during the animal cell passage. Using pZ189 and other shuttle vectors, this laboratory has extensively characterized the types of mutations that occur in mammalian cells either spontaneously or in response to DNA damage. Mutational specificity has been demonstrated for UV and the benzpyrene derivative, and analysis of the sequence specificity of these mutations has led to models that explain how the mammalian DNA polymerase introduces errors during DNA synthesis. During the past year, studies with pZ189 in an *in vitro* DNA replication system have indicated that certain cellular factors, in addition to the DNA polymerases, influence replication fidelity, and these cellular factors are now being characterized. Workers in this Laboratory have also shown that an increased mutational frequency is induced by general stressing agents such as phorbol esters, with important implications for the measurement of cumulative risk from carcinogens and other DNA damaging agents. Moreover, for the first time, it has been unequivocally shown that a system exists in mammalian cells which appears to be analogous to the "SOS" (inducible repair and mutagenesis) pathway of bacteria. This laboratory also investigates DNA repair processes, which play an important role in protecting cells against the mutagenic and carcinogenic action of toxic agents. With the use of virus-based expression vectors *in vivo* and *in vitro*, the molecular mechanisms by which DNA lesions are repaired in mammalian cells are being characterized. These repair functions are enhanced after DNA damage, and enhanced repair is accompanied by the induction of lesion-specific DNA binding proteins. During the past year, one such protein was isolated and purified to homogeneity. It is the first DNA damage-inducible, damage-specific DNA binding protein to be identified, and significantly, it appears to be absent in the cells of at least one group of patients with xeroderma pigmentosum, a disorder characterized by susceptibility to skin cancer at an early age and known to be due to defective DNA repair. With regard to studies on DNA replication, the major emphasis is on certain oncogene and anti-oncogene interactions, which stimulate or inhibit cell proliferation. These relationships are being pursued in an SV40-induced hamster osteosarcoma model (human osteosarcoma is known to be associated with a deletion of the anti-oncogene, *Rb-1*). The molecular interactions between the T antigen oncogene of SV40 and the hamster homolog of *Rb-1* are under investigation.

The **Laboratory of the Deputy Scientific Director** is concerned with the mechanism of action of nerve growth factor (NGF). It is a typical peptide growth factor, structurally related to certain oncogene products, and responsible for differentiation of certain types of nerve cells. NGF binds to neuronal receptors, initiating a cascade of intracellular events, which eventuate in the expression of specific genes. This group has been instrumental in elucidating the events that mediate the actions of NGF, relying on studies of the NGF responsive cell line PC12. The laboratory has found that NGF binding is quickly followed by an increase in cAMP, phosphoinositide metabolism, and arachidonic acid release. These changes then lead to alterations in various cellular kinases and to the phosphorylation of several critical proteins, including elongation factor II, the ribosomal S6 protein, and a nuclear protein that may be involved in regulating the transcription of specific genes. Work in cell-free systems suggests that a number of phosphorylation cascades are induced in parallel by NGF binding to its receptor, and the search is on for the single mechanism that couples the receptor to these various cascades. Several oncogenes, e.g., *src* and *ras*, may modulate NGF activity, and these interactions are being dissected in appropriate systems. Specific genes, the expression

of which underlies NGF-induced differentiation, are also being identified, including the gene encoding the neuron-glia recognition molecule, N-CAM. Interestingly, NGF-induced expression of N-CAM is inhibited by glucocorticoids, which cause a down regulation of the NGF receptor. During NGF induced differentiation, the expression of other genes is decreased such as, for example, the gene which encodes calpain, a calcium activated intracellular protease. This group has recently shown that the decrease in calpain activity is caused by an increase in the activity of the endogenous calpain inhibitor, calpastatin, and these changes may be directly involved in the generation of neurites. Interestingly, NGF and EGF have inverse affects upon PC12 cells, with EGF promoting cell proliferation and NGF promoting differentiation. These investigators have previously found that NGF induces down regulation of EGF receptors, and in the past year, they have gathered evidence that the decrease in EGF receptors is probably due to an NGF-induced alteration in their phosphorylation state. Further study of these interactions between NGF and EGF should yield important insights into the control of differentiation and cell division.

The Institute continues to acquire additional laboratory space. During the past year, we gained 25 additional modules for neurobiology research within the neuroscience complex (Buildings 36 and 37). Also during 1989, an addition to Building 6 was constructed ("Building 6B"), which will soon be ready for occupancy. Building 6B will house one of the world's largest transgenic mouse facilities, as well as 10,000 square feet of additional laboratory space for the Laboratory of Molecular Genetics and the Laboratory of Mammalian Genes and Development. Construction was also begun on Building 49, a major new facility in the center of the NIH campus, which will allow continuing expansion of our neuroscience and neuroendocrinology research. This building will also house the neuroscience laboratories of several other Institutes as well as a state-of-the-art primate facility. Finally, we completed the renovation of Building 32 for the Cell Biology and Metabolism Branch, which has allowed a further expansion of that Laboratory's research. Still another small building will be renovated during the coming year for this Branch, mainly housing an advanced electron microscopy suite. We anticipate that the NICHD intramural program will have grown from 41,000 square feet of laboratory space (in 1985) to approximately 110,000 square feet by 1991.

In major senior staff changes during the past year, Dr. Mark L. Mayer, who studies the structure and function of excitatory neurotransmitter receptors, was granted tenure in the Laboratory of Developmental Neurobiology, and Dr. Thomas Sargent, who is investigating *Xenopus* regulatory DNA sequences and their binding proteins, was granted tenure in the Laboratory of Molecular Genetics. Dr. William Gahl was appointed Chief of the Human Genetics Branch. Dr. Gahl is the author of landmark work defining the basic defect in cystinosis, an important human inborn error of metabolism, as an abnormality in carrier-mediated transport of cystine across the lysosomal membrane. Gahl has also led a national study on the treatment of cystinosis with cysteamine, a drug which he believed would be very useful on the basis of his elucidation of the mechanism of this disorder. Cystinosis is the first lysosomal storage disease for which a directed treatment is effective. In 1990, Dr. Gahl will also become Director of the NIH-wide Interinstitute Genetics Training Program. A new department was created within the Institute, the Laboratory of Mammalian Genes and Development, and its first chief is Dr. Heiner Westphal. Dr. Westphal is one of the country's most noted molecular biologists, with widely recognized work earlier in his career on the molecular genetics of the DNA viruses, SV40 and adenovirus, as well as on RNA processing in eukaryotic cells. Recently, his Laboratory has focused on the biology of transgenic mice, and using this system, he was among the first to establish that developmental and tissue-specific expression of genes depends on interactions between DNA binding proteins and promoter sequences that convey temporal and spatial specificity. In 1990, another new Laboratory will be created within the Institute, the Laboratory of Molecular Growth Regulation, and its first chief will be Dr. Bruce Howard, who is transferring to the NICHD from the National Cancer Institute. Drs. Daniel Nebert and Kathleen Dixon left the Institute to become division directors in the Institute of Environmental Health at the University of Cincinnati. With Dr. Nebert's departure, the Laboratory of Developmental Pharmacology is being vacated. Drs. Richard Sherins and Frank A. Pedersen retired from their positions in

the Developmental Endocrinology Branch and Laboratory of Comparative Ethology, respectively. Drs. Bruce Nisula and Fernando Cassorla were named Acting Chief of the Developmental Endocrinology Branch and Acting Clinical Director, respectively, in anticipation of the 1990 move of Dr. Lynn Loriaux to the University of Oregon.

During 1989, a number of Sections were created within established Laboratories and Branches. In the Laboratory of Comparative Ethology, a Section on Social and Emotional Development was established under the direction of Dr. Michael Lamb. Research in this section is focused on the factors affecting psychosocial and emotional adaptation in infancy, childhood and adolescence, in a variety of cultural and social contexts. Dr. Mark Bornstein was appointed head of the Section on Child and Family Research, which investigates the early development of behavior and personality within the context of various family structures. In the Laboratory of Molecular Genetics, a Section was established on the Molecular Genetics of Lower Eukaryotes, directed by Dr. Alan Hinnebusch. This Section focuses on the regulation of amino acid biosynthesis in yeast as a model of "global" genetic control pathways in eukaryotes. In the Developmental Endocrinology Branch, a Section on Pediatric Endocrinology was established, directed by Dr. George Chrousos. This group studies the regulation of hypothalamic-pituitary-adrenal function and its role in stress, metabolism, reproduction and immune regulation. Dr. Chrousos was also appointed to lead the Pediatric Endocrinology Training Program.

Our clinical research fellowships for physicians being trained in investigative endocrinology or medical genetics continue to thrive, and in the past year, we developed new sources of support for postdoctoral (Ph.D. and M.D.) Fellows who are receiving advanced training in basic science. These mechanisms feature postdoctoral stipends paid by the Institute, but which do not encumber regular government positions. These new fellowships are greatly enhancing our ability to offer postdoctoral training, especially for American graduates, and include an intramural National Research Scholarship Award for physicians with little prior laboratory training; a fellowship in biotechnology administered for us by the National Research Council; and a new program that is intended for American postdoctoral candidates (Ph.D. or M.D.) in any of the biological sciences. The latter mechanism (Intramural Research Training Award) is the domestic equivalent of our long-standing Visiting Fellow program for postdoctoral candidates from abroad. Together with NIMH, we are also about to launch a new program for the support of intramural training in the neurosciences, to be administered by the National Research Council. This neuroscience fellowship will accept its first applicants early in 1990. We have also been successful in identifying new donors of stipends, including endowments by private industry and foundations. NICHD funds administered by the NIH's Fogarty International Center have supported sabbatical visits by senior scientists from abroad under the terms of formal bilateral agreements between the NIH and institutions in Europe, Latin America, Asia, and the Middle East. Finally, a number of medical students supported by the Howard Hughes Medical Research Institute at the NIH are working in our laboratories during an elective year. During 1989 we also initiated a new fellowship for pre-doctoral students. This program is designed for Ph.D. candidates who have completed their formal course work in a university, will receive their degree from that university, and who will be permitted to undertake their thesis work in an intramural laboratory. Our summer student program was extremely successful during 1989, with more than 60 undergraduate, graduate, and medical students working in our Institute.

Peer review of intramural research, conducted by the Institute's Board of Scientific Counselors and *ad hoc* experts, continues to receive great emphasis, with rigorous three-day site visits to each Laboratory at four-year intervals (as well as a less detailed twice-yearly review of the Intramural Program in its entirety). During 1989, visits were made to the Human Genetics Branch and to the Laboratory of Developmental and Molecular Immunity, with detailed critiques prepared as a result of these visits. The membership of the Board of Scientific Counselors reflects the increasing diversity of research interests within this Intramural Program, and currently includes Joseph G. Gall, Ph.D. (Chairman), Senior Member, Carnegie Institution (Baltimore); Stanley Cohen, Ph.D., Professor of Biochemistry, Vanderbilt University; Joseph J. Campos, Ph.D., Director, Institute of Human Development,

and Professor of Psychology, University of California at Berkeley; Peter O. Kohler, M.D., President, Oregon Health Sciences University; Lynn T. Landmesser, Ph.D., Professor, Department of Physiology and Neurobiology, University of Connecticut; Stanley G. Nathenson, M.D., Professor of Microbiology and Immunology, Albert Einstein College of Medicine; Alan L. Schwartz, Ph.D., M.D., Professor, Departments of Pediatrics and Pharmacology, Washington University School of Medicine, St. Louis; Savio L.C. Woo, Ph.D., Professor of Cell Biology and Molecular Genetics, Baylor College of Medicine; and Neena B. Schwartz, Ph.D., William Deering Professor of Biological Sciences, Department of Neurobiology and Physiology, Northwestern University.

Other developments in the past year include the continued strengthening of all aspects of the use of animals employed in our research; the achievements of our veterinary program, directed by Dr. William Stokes, were recognized by the creation of a new section on the Management of Research Animals. Dr. Stokes will also be the NIH's lead veterinarian for the new Building 6B shared animal facility, which is the largest and most specialized interinstitute facility. We have continued to develop new computer-based administrative procedures in the Office of the Scientific Director, so as to maximize the efficiency with which our resources are deployed. These new administrative approaches are ensuring the maximum yield with respect to scientific productivity while the current climate of constrained resources persists. A number of laboratories within the program are involved in various aspects of research on AIDS; they competed successfully for 1989 special set-aside funds for AIDS research above the Institute's normal budget ceiling, and it is likely that this budget increment will be continued for the foreseeable future.

Seminars by the twelve Laboratories and Branches in this Program were numerous and well attended throughout the year, such that this Institute organized a relatively large fraction of the NIH's overall offering of intramural seminars and workshops. During the past year also, a number of major research conferences were organized by NICHD scientists, with international participation, including: "Calcium Metabolism: Issues in Absorption and Kinetics," hosted by the Laboratory of Theoretical and Physical Biology; "Regulation of Testicular Function: Signaling Molecules and Cell-Cell Communication," hosted by the Endocrinology and Reproduction Research Branch; "New Approaches to the Interface of Field and Laboratory Primate Research," hosted by the Laboratory of Comparative Ethology; "Genetic Response to Environmental Adversity," hosted by the Laboratory of Developmental Pharmacology; "Social and Psychological Stresses on the Immune System in Primates," hosted by the Laboratory of Comparative Ethology; "Suprachiasmatic Nucleus: The Mind's Clock," hosted by the Laboratory of Developmental Neurobiology; "Biochemistry, Molecular Biology and Physiology of Phospholipase A2 and its Regulatory Factors," hosted by the Human Genetics Branch; and "Polycystic Ovarian Syndrome," hosted by the Developmental Endocrinology Branch.

During 1989, the NICHD was especially honored by the conferring of an honorary doctorate upon Dr. Igor Dawid by the University of Lausanne, in recognition of the profound influence Dawid's career has had on our understanding of gene regulation, especially during embryonic development. Dr. Dawid, who is Chief of the Laboratory of Molecular Genetics, continues as Chairman of the Editorial Board of the Proceedings of the National Academy of Sciences, and during the past year, he was elected to membership in the American Academy of Arts and Sciences. During the past year also, the Society for Neuroscience chose Dr. Mark Mayer to receive its prestigious Young Investigator Award for his ground-breaking work on the structure and function of neuronal NMDA receptors. These receptors appear to be critical for memory and learning, but they are also implicated in the damage that occurs during cerebral anoxia. Dr. John Robbins, Chief of the Laboratory of Developmental and Molecular Immunity, was awarded the Public Health Service Distinguished Service Medal, the highest departmental honor, for his many contributions to vaccine development, which have been of great significance to the public health. During the past year, Dr. Robbins was also invited to deliver both the Louis Weinstein Lecture at Tufts University and the Maxwell Finland Lecture of the Infectious Disease Society. Dr. Richard Klausner, Chief of the Cell Biology and Metabolism Branch, received a number of honors during the year, including an

invitation to deliver the Presidential Lecture of the Endocrine Society at its annual meeting, in recognition of his fundamental contributions to our understanding of RNA regulatory elements and their role in the control of intracellular iron levels. Klausner was also appointed to the Scientific Advisory Committee of the Weizmann Institute (Israel) and he delivered a Plenary Lecture at the annual meeting of the American Society for Cell Biology. Also during 1989, Dr. Heiner Westphal, Chief of the Laboratory of Mammalian Genes and Development, served as Guest Professor at the University of Basel, an honor bestowed only once every five years. Drs. George Chrousos and Gordon Cutler were elected to membership in the American Society of Clinical Investigation, and Dr. Mark Bornstein was elected as a Fellow in the American Association for the Advancement of Science. Dr. Yoke Peng Loh was awarded the PHS Superior Service Award for her work on the processing of pro-opiomelanocortin, and Dr. D. Lynn Loriaux received the PHS Meritorious Service Medal for his many contributions in the area of developmental endocrinology. Finally, Dr. Arthur Levine was invited to be the founding editor of a new journal devoted to cellular and molecular biology, *The New Biologist*, and the first issue of this journal was published in October. Additionally, many of the Institute's senior investigators held honorary lectureships and visiting professorships during the year, and also served as chairmen of such major meetings as Gordon Conferences.

This Institute continues to mount an ambitious intramural program in the general area of developmental biology, with a continuing increase in the quality and quantity of its scientific productivity even as the rate of growth in research support has declined. A rank order survey of the various NIH Institutes with respect to their yearly number of publications in nine of science's most prestigious journals, normalized to Institute budget, includes the NICHD among the top three. The program's stature is well reflected not only in the honors and awards accruing to its senior investigators, but in the new resources allocated to the program by the NIH and by the Congress.

Arthur S. Levine, M.D.  
Scientific Director



## LABORATORY OF MOLECULAR GENETICS

Igor B. Dawid, Ph.D., Chief



Left to right: J. Levin, R. Crouch, T. Sargent, M. Cashel, J. Kennison, I. Dawid, R. Weisberg, K. Mahon, A. Hinnebusch, H. Westphal

During the past year the Laboratory of Molecular Genetics continued in its efforts to analyze mechanisms of the transmission, recombination and expression of the genome in a variety of organisms. These range from microorganisms to complex animals. Bacteria, yeast, and several viruses have been a focus of study. Molecular genetics originated by the study of these microorganisms, and they continue to be the most effective systems for the analysis of basic biological mechanisms. Two major programs in this area are concerned with the control of the metabolism of *Escherichia coli* and yeast by the environment. The complex networks that mediate this control are excellent models of regulatory mechanisms in all of biology.

An important emphasis in the Laboratory was placed on the study of developmental processes in different animal systems, such as *Drosophila* and *Xenopus*. Modern developmental biology has focused on these two animals for several reasons. Classically, the amphibian embryo has been the model of choice, because, as a vertebrate, its embryogenesis is similar to that in mouse or human, but early stages are far more accessible for manipulation and biochemical study. Thus, studies on the initial establishment of the embryonic axes and the germ layers are effectively carried out in the amphibian embryo. The animal that combines almost all advantages for a molecular genetic study of development is the fruit fly *Drosophila melanogaster*. The most comprehensive genetics in any animal, combined with extensive studies on embryogenesis, cell lineage and, recently, molecular biology combine to make *Drosophila* development the best understood in the animal kingdom. Major insights, valid in all animals, have been obtained through the study of this organism.

The degree to which insights in one animal can be translated to another is one of the most exciting developments of recent years. Related genes are used for similar regulatory purposes

in quite different animals, and structural motifs are found to recur in different proteins, utilizing a functional entity in different context. Two findings exemplify this "unity of life" theme better than most others: the homeobox and the zinc finger. Both are specific DNA-binding motifs that recur in numerous transcriptional regulatory proteins, many of which have key control functions in development. The homeobox and zinc finger were discovered initially in *Drosophila* and in *Xenopus*, respectively, and have since been found in all eukaryotic organisms. Work in the Laboratory in the past year has added substantially to our understanding of the distribution, structure and function of members of these protein families. Especially exciting is the discovery in *Xenopus* of a homeobox gene that is responsive to mesoderm induction signals and whose function in the embryo appears to be restricted to the gastrula stage.

The research group headed by **Michael Cashel** has pursued the question how a bacterial cell regulates its gene expression during transitions between balanced growth and nutritional impoverishment. These researchers are particularly interested in the regulatory role played by the nucleotide, guanosine 3',5'-bipyrophosphate (ppGpp), as an intracellular signal to control these processes in *E. coli*. To analyze this question, they have dissected the genetic elements controlling ppGpp synthesis (the *relA* gene) and ppGpp degradation (the *spoT* gene). Each gene, obtained in plasmids, was localized, sequenced, precisely deleted, and replaced by an antibiotic resistance gene. These null alleles were placed in the chromosome by manipulations involving crossing the modified genes into a phage  $\lambda$ , followed by preparing and curing phage lysogens to allow quantitation of recombinational replacement.

The results are both surprising and complex. Two routes of ppGpp synthesis have been established. One route was previously known to be associated with the RelA protein operating on a ribosome paused on an mRNA codon for lack of an available aminoacylated tRNA. When a *relA* deletion eliminates this route, a second route of synthesis persists, which responds only to energy source deprivation. Unexpectedly, the second route of ppGpp synthesis is abolished when the *spoT* gene is deleted, along with the *relA* gene, yielding the first prokaryotic cell devoid of detectable ppGpp. Thus, the *spoT* gene is either bifunctional and encodes, in addition to a ppGpp degrading activity, a ppGpp synthetic function, or *spoT* represses a second (still unidentified) synthetic gene. There are reasons to believe that a bifunctional protein is more likely.

Cells lacking ppGpp give clues to the functions of ppGpp. Such cells are found to be vulnerable to physiological stress; normally innocuous transient deprivations of either energy sources or amino acids become lethal, as does thermal shock. In contrast, transitions from nutritionally poor to enriched conditions occur normally. Surprisingly, these cells also have cell division defects as well as growth requirements for as many as 15 of the 20 amino acids. Single step revertants that grow in the absence of amino acids have been localized to the RNA polymerase  $\beta$  subunit, showing that the auxotrophic phenotype can be overcome by modification of transcription, and that RNA polymerase is a likely target of ppGpp regulation.

The group headed by **Robert Crouch** is concerned with the structure and function of ribonuclease H (RNase H), an enzyme that degrades an RNA strand only when it is complexed with a DNA strand. Earlier work has demonstrated that *E. coli* without RNase H activity cannot survive mutations in certain recombination genes. These and other studies suggest that RNase H is important in repair and recombination. The remarkable specificity of the enzyme for the RNA strand in an RNA/DNA hybrid makes a detailed structural analysis particularly interesting. Therefore a crystallographic study of RNase H was initiated. RNase H of *E. coli*, containing either methionine or selenomethionine, has been produced and crystallized. Crystals of the methionine containing RNase H have been analyzed by usual methods and shown to be in the space group  $P2_12_12_1$ , with cell constants of  $a=41.99\text{\AA}$ ,  $b=86.68\text{\AA}$  and  $c=36.88\text{\AA}$ . Crystals of the selenomethionine containing RNase H have been obtained and data collected by Multiwave Anomalous Dispersion, a technique based on high energy X-rays.

The retrovirus *pol* gene encodes a protein that has reverse transcriptase and RNase H activity. The retroviral RNase H has been studied in collaboration with Dr. Levin. Retroviral RNase H has long been thought to be an exonuclease. The enzymatic nature of the RNase H has been reexamined on certain well defined substrates. RNase H can degrade RNA of RNA:DNA hybrids even when the DNA is more than 200 nucleotides from either the 3' or 5' termini. This result is consistent with the fact that the same hybrids can serve as primers for DNA synthesis, but is at odds with the idea that the RNase H acts in an exonucleolytic manner. Further proof that RNase H acts endonucleolytically was obtained by constructing a circular RNA of about 170 bases, which was annealed to a complementary 20 base oligomeric DNA. This substrate is also cleaved by the retroviral RNase H.

A group headed by Igor Dawid is concerned with molecular mechanisms during early amphibian development. As in all vertebrates, the frog embryo generates its different tissue distributions in an orderly process through a series of cell interactions called embryonic induction. The profound influence of induction on the developmental fate of embryonic cells had been known for many years, but only recently has there been progress in analyzing the molecular basis of these events. A role for growth factors, specifically of the TGF- $\beta$  family, has been suggested in mesoderm induction. Using the mesoderm inducing factor (MIF) from XTC cells, the activation of several immediate early genes in the responding tissue has been demonstrated. One of these genes encodes a homeodomain protein and was named *Mix.1*; it is expressed in the vegetal region during a short period in blastula and gastrula embryos, and may be involved in the establishment of mesoderm and endoderm.

In a related project, the development of the nervous system in *Xenopus* was studied by the isolation of neurospecific genes that are activated during gastrula stages. Five genes in this class were identified by sequencing. They include a  $\beta$ -tubulin, a homolog of the  $\beta$ -subunit of Na/K ATPase, and a gene encoding a putative RNA-binding protein. Especially the latter finding is of considerable interest, because RNP proteins may be involved in RNA processing and transport, raising the possibility of studying tissue-specific control of RNA metabolism in the brain.

The other major DNA-binding motif, in addition to the homeodomain, is the zinc finger. Both structural studies of the original finger protein, *Xenopus* TFIIIA, and an analysis of finger-containing genes in the human genome were undertaken. Preliminary evidence was obtained that the genome contains many such genes, at least 40 and possibly well over 100.

Using the same model system, *Xenopus laevis*, the group headed by Tom Sargent aims to identify informational molecules that are localized in the egg, thereby assigning different fates to different regions in the early embryo. Such localized informational molecules have been postulated since the last century, but direct evidence for their nature has been lacking. To study this issue, Dr. Sargent and his colleagues attempt to characterize proteins that regulate gene expression in vertebrate embryos in a regionally specific manner. The primary model system is based on the transcriptional regulation of an epidermal keratin gene, *XK81A1*, in *Xenopus* embryos. The *cis*-regulatory elements of this gene have been mapped using transgenic frog embryos as an assay system. One regulatory site has been precisely located and characterized in detail. A positive transcription factor, KTF-1, binds to this DNA domain and stimulates the activity of the keratin promoter. Characterization of KTF-1 and cloning of the gene encoding this factor are currently being pursued, and other positive and negative regulatory elements in the keratin promoter are being investigated.

In addition, other developmentally interesting genes have been recovered from two subtracted cDNA libraries prepared as part of this program: *UVS.1* and *UVS.2* (for ultraviolet sensitive) are expressed specifically in cement gland and hatching gland, respectively. These and other genes in this series are markers for dorsoanterior axis formation. The gene *DG72* is expressed specifically in the posterior region of the embryo from gastrulation through early organogenesis. This gene is inducible with the mesoderm inducer XTC-MIF, and the predicted protein sequence includes a region similar to the "zinc finger-like" RNA binding

domain of retroviral gag proteins, as well as an extended acidic helix with a short "leucine zipper". The regulation and function of these genes are the subject of ongoing investigation.

Three groups are interested in the molecular genetics of *Drosophila*. Work directed by Igor Dawid is concerned with the homeotic regulatory genes *fsh* and *trithorax* (*trx*). The *fsh* gene is a maternal effect locus, which leads to homeotic transformations in the progeny of mutant females; the frequency of transformations is greatly increased by interaction between mutations in *fsh* and *trx*. The major products of *fsh* have been identified as two large proteins with membrane-spanning domains. Antisera produced against fusion proteins show that the primary translation products are processed to three smaller proteins, and preliminary evidence for glycosylation of the *fsh* proteins has been obtained. Analysis of progeny of *fsh* mutant females shows distorted expression in gap, segmentation and homeotic genes, the probable basis for the developmental abnormalities in these embryos. In particular, the domain of expression of the gap gene, *Krüppel*, is diminished, showing a very early effect of maternal *fsh* deficiency on embryonic gene expression.

The *trithorax* (*trx*) gene, a major regulatory locus in *Drosophila*, has been cloned previously. Sequence analysis of cDNAs predicts a very large protein of molecular mass 400,000 as the major product of *trx*. This protein contains cysteine-rich regions that are hypothesized to be zinc-dependent nucleic acid-binding domains.

Studies carried out by Susan Haynes have focused on the genes encoding RNA-binding proteins in *Drosophila*. Such proteins are of great interest, since they may be involved in RNA processing and transport, offering opportunities for regulation of gene expression in the cell. Evidence from various sources has accumulated in recent times that such control is important in development and cell physiology. Haynes' work has identified a family of genes encoding hnRNP proteins, the *Hrb* family, in *Drosophila*. Two members of this gene family have been analyzed in detail. The *Hrb98DE* locus produces eight different RNAs by alternative splicing. This gene is expressed throughout development, but the contributions of the different RNAs vary. The *Hrb32AB* locus gives rise to two RNAs by the use of different polyadenylation sites. Antisera produced against fusion proteins detect multiple bands in fly extracts. The antigens comigrate with nuclear RNA in sedimentation analysis, as predicted for hnRNP proteins. Ongoing work is aimed at generating mutations in the *Hrb* loci for an analysis of the *in vivo* functions of these genes and their products.

The group headed by Jim Kennison is interested in the genetic control of establishment of the body plan in *Drosophila*. Genes involved in the determining segmental identity have been identified on the basis of genetic interactions with homeotic mutations, mutations already known to affect the process. Of the eighteen genes identified by interacting mutations, three act as negative regulators of homeotic genes, and the remaining fifteen appear to be positive regulators, ancillary factors, or targets of homeotic gene function. Of the latter fifteen, the genes *kismet* and *Sex combs reduced* behave as targets for the homeotic gene *Antennapedia*. The genes *brahma*, *osa*, *moira*, *skuld*, *kohtalo*, and *sallimus* behave as ancillary factors for *Antennapedia* function, with at least *brahma* also acting as a positive regulator of *Antennapedia* gene function. Mutations associated with insertions of a *Drosophila* transposable element have been isolated for the genes *kismet*, *brahma*, and *osa*. The sequence of the putative protein product of the *brahma* locus has been determined.

A 55 kilobase region of the genome including the *brahma* locus has been characterized by molecular and genetic methods. In addition to the *brahma* locus, three transcription units have been identified within this region of DNA. Sequence analyses of cDNAs from two of the three transcription units predict putative protein products with extensive sequence similarities to mammalian proteins thought to be involved in signal transduction. Thus, one of these putative protein products has similarities to the catalytic subunit of cAMP-dependent protein kinase, while the other product is similar to a GTP-binding protein termed ARF (ADP-ribosylation factor). Approximately thirty-five mutations in essential genes in this chromosome region have been isolated. These mutations identify at least three genes in addition to the *brahma* locus.

**Alan Hinnebusch** and his colleagues are involved in a program to study the elaborate regulatory network that allows yeast cells to respond to the availability of amino acids in the medium. When amino acids are lacking, the cells turn up the production of the various enzyme systems that synthesize amino acids. However, when these nutrients are plentiful in the medium, it is of considerable selective advantage for the cell to turn off the production of such enzymes, which would be a waste of resources under these conditions. A hierarchy of regulatory genes mediates this control in yeast; various mechanisms are involved, including transcriptional and translational regulation and protein modification by kinase action. The proximal factor in the hierarchy is *GCN4*, a transcriptional activator of amino acid biosynthetic genes, which is itself regulated at the translational level by short open reading frames (uORFs), present in the leader of *GCN4* mRNA. The mechanism of translational regulation of *GCN4* expression has been a major focus of research of this group, both because of its intrinsic interest and as an example of translational regulation in eukaryotes in general. *GCN4* expression is repressed in nonstarvation conditions by uORFs 3 and 4, whereas uORF1 stimulates *GCN4* expression by overcoming the inhibitory effects of uORFs 3 and 4. The distinct functions of uORFs 1 and 4 are determined by sequences immediately surrounding their stop codons, suggesting that translation termination occurs differently at these two sites. Reinitiation after uORF4 translation is very inefficient in nonstarvation conditions; by contrast, uORF1 translation is compatible with downstream initiation, and this property is important for its ability to regulate uORF4. Translation of uORF1 may alter the ribosome in a way that facilitates its advance through uORF4 sequences in amino acid-starved cells. Positive and negative *trans*-acting factors are also required to regulate ribosome movement in the *GCN4* mRNA leader. Modulating the activity of the general translation initiation factor eIF-2 appears to be one important function of the positive regulators *GCN2* and *GCN3*. At least two negative regulators of *GCN4* expression, *GCD1* and *GCD2*, are essential factors that probably also function in translation initiation. Interestingly, *GCD1* and *GCD2* appear to exist in a complex with *GCN3*, suggesting that *GCN3* directly modulates the activity of these gene products. *GCN2* stimulates *GCN4* expression by functioning as a protein kinase. Homology between the C-terminus of *GCN2* and histidyl-tRNA synthetases suggests that *GCN2* detects amino acid starvation by directly monitoring the concentration of uncharged tRNA. In this model, tRNA binding stimulates *GCN2* kinase activity. Mutations in *GCD* genes, in genes encoding subunits of eIF-2, and in *GCN3* have been identified that all bypass the requirement for *GCN2* to derepress *GCN4* expression, making these factors potential substrates for phosphorylation by *GCN2* in amino acid-starved cells.

Ribosomal protein (rp) genes are repressed under the same amino acid starvation conditions that lead to elevated expression of amino acid biosynthetic genes under *GCN4* control. Repression of the *RPL16A* rp gene requires regulatory sequences that include the binding site for the transcriptional activator TUF. Regulation of *RPL16A* expression occurs independently of *GCN* factors, indicating that separate pathways exist for these two transcriptional responses to amino acid starvation.

The goal of the program headed by **Judith Levin** is to define the molecular mechanisms involved in the replication of mammalian retroviruses and in particular, to understand the factors that influence the regulation and expression of viral genetic information. Studies are being carried out with the murine leukemia virus system (MuLV). Current interest is focused on the organization of the reverse transcriptase coding region and on the identification of functionally significant sequences within the RNase H domain. Molecular clones containing reverse transcriptase sequences are being expressed in *E. coli*. These clones include one that contains the entire coding region, as well as constructs in which the gene has been modified by deleting (i) the RNase H domain; (ii) the polymerase domain; and (iii) sequences within or immediately upstream of the RNase H domain. The results confirm the previous assignment of the RNase H domain to the carboxyl-terminal region of reverse transcriptase. Thus, RNase H activity is lost by deleting all or a substantial portion of this region, but is retained when the deletion is in an upstream sequence. Studies are in progress to determine the polymerase activity of these constructs and to evaluate the functional relationship between the polymerase and RNase H domains.

A second project of this group deals with translational control of viral gene expression. In retroviruses, termination codons are found at the *gag-pol* junction, which do not, however, lead to a complete stop of translation at this position. Different mechanisms, such as read-through suppression and frame shifting, occur in different viruses to allow the modulated continuation of translation through this region. Dr. Levin and her colleagues have focused on the role of nucleotide context in suppression of the in-frame UAG codon at the MuLV *gag-pol* junction. A short 66-base sequence, consisting almost entirely of nucleotides downstream of the termination codon, has been shown to provide a sufficient context for readthrough suppression. In addition, *in vitro* and *in vivo* experiments, showing suppression with constructs containing UAA or UGA instead of UAG, indicate that (i) the signal inducing suppression is not unique for UAG; (ii) mammalian cells and cell extracts contain tRNAs capable of translating UAA and UGA as amino acids; and (iii) UAA is not an absolute termination signal in higher eukaryotes (as originally thought). Experiments to identify the precise signals utilized by the virus to mediate suppression, including the potential role of mRNA secondary structure and the involvement of ribosomal pausing in misreading of the termination codon by suppressor tRNA, are underway.

Robert Weisberg and his colleagues have continued their interest in the study of gene expression and recombination, using the lambdoid bacteriophages as their system. As a result of extensive studies by many laboratories, our understanding of the molecular mechanisms controlling the life cycle of this group of viruses may be the best understood of any biological entity. Recent work in Dr. Weisberg's group has focused on the comparative analysis of gene expression and recombination in the related phages HK022 and  $\lambda$ . Gene expression in the temperate coliphage HK022 is similar to that in other lambdoid phages: the genes that are expressed early after infection are organized into two divergently transcribed operons, and the initiation of transcription from the corresponding promoters (pL and pR) is blocked by the phage repressor. However, HK022 early transcription also has novel features. First, the two early operons are transcribed poorly when phage DNA synthesis is blocked. Since pL and pR are strong promoters *in vitro*, it appears that some factor or condition missing *in vitro* limits transcription *in vivo*. Second, HK022, in contrast to its relatives, does not encode a factor that promotes antitermination of early transcription. Nevertheless, antitermination of early transcription occurs in HK022, and requires specific sites located in the early operons. Thus, antitermination in this phage is either factor-independent or the required factors are encoded by the host. Third, HK022 expresses *nun*, the first gene of the pL operon, in the presence of phage repressor. *Nun* transcription in these conditions originates not at pL but at an upstream promoter. The *nun* protein protects HK022 lysogens from superinfection with phage  $\lambda$  by terminating transcription at the  $\lambda$  *nut* sites. The specific *nut* sequences required for *nun* termination overlap but do not completely coincide with those required for antitermination promoted by the  $\lambda$  N protein.

HK022 and  $\lambda$  both encode proteins that promote recombination between special DNA sequences, called attachment sites. The mechanism of site-specific recombination in the two phages is very similar, but the sites and one of the proteins (the Int protein) are not interchangeable. To localize the determinants that distinguish these two recombination systems, hybrid *int* genes were constructed and attachment sites and their function analyzed. The determinants of protein specificity are located amino terminal of Int residue 281. This segment does not contain the highly conserved "recombination domain" of the Integrase family proteins. The specificity determinants of the attachment sites are located in the regions previously identified as "core type" Int binding sites in  $\lambda$  by Ross and Landy. The segments corresponding to the C and B'  $\lambda$  core Int binding sites appear to be especially important, and the C' and B sites to be less important. The arms of the phage attachment site, which contain IHF, Xis and "arm-type" Int binding sites, do not determine the specificity difference.

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## LABORATORY OF MAMMALIAN GENES AND DEVELOPMENT

Heiner Westphal, M.D., Chief



Left to right: K. Mahon, H. Westphal

The goal of this newly established laboratory is to understand fundamental questions of development, differentiation and oncogenesis in man. The mouse is a key model for human developmental biology, in view of the great similarity of embryogenesis in all mammalian species. It is also the most suitable mammal for genetic studies, because of the numbers that can be handled. Many techniques for embryo manipulation and gene transfer have been established in this animal model, and have greatly accelerated progress in this field. **Heiner Westphal**, Kathy Mahon and their colleagues have utilized these techniques in several productive ways to gain insights into mechanisms of gene control in the intact mammalian organism. In particular, they have identified spatial and temporal patterns of expression of a number of genes that are thought to be important for early development, have measured the effects of oncogene products in transgenic mice and are exploring the technological potential of transgenic animals.

One of a number of projects addresses questions of early embryonic development. Recently, attention has been drawn to several gene families bearing conserved DNA binding motifs, suggestive of a role in transcriptional regulation. Of these, the best candidates for a developmental role are the homeobox genes, largely because of their extreme conservation among animal phyla and their important function in the development of the fruit fly, *Drosophila melanogaster*. Members of the team have investigated the spatial pattern of expression of two members of the *hox* homeobox gene family from the mouse, *hox 1.1* and *hox 1.5*, by *in situ* hybridization during mouse embryogenesis, and have found that each is expressed in distinct, yet overlapping, regions of the neural tube and somites during the formation of the embryonic axis. The results are consistent with the notion that the *hox* genes function to specify positional identity within regions along the axis. The transgenic mouse system will be utilized to test this hypothesis more directly. Transcripts of individual

*hox* genes are not detectable until after gastrulation is well underway, and accordingly do not appear to play a role in major early decisions, such as the establishment of antero-posterior and dorso-ventral axes, initiation of morphogenetic movements of gastrulation, and mesoderm or neural induction.

Since such very early determinative events in *Drosophila* are orchestrated by homeobox genes, many of which contain divergent homeobox sequences, it seems likely that there are more genes yet to be identified in the mouse that could be involved in these early decisions. To identify such genes, degenerate homeobox oligonucleotide probes are being used to screen mouse genomic and cDNA libraries. In addition, attempts are being made to identify the mouse homolog of *mix 1*, a homeobox containing gene isolated from *Xenopus* by Drs. Rosa and Dawid (LMG), which may play a regulatory role in induction.

Another class of conserved genes under scrutiny is the *myc* gene family. A conserved DNA binding domain, encoded by *myc* proto-oncogenes, has also been found in several genes known to be important in development: *MyoD*, which is involved in myogenesis in the mouse; two genes of the *achaete-scute* complex, involved in *Drosophila* neurogenesis; and *twist*, a gene involved in establishing the germ layers in *Drosophila*. Thus, the *myc* genes, originally defined as oncogenes, may have a regulatory role in normal development. To investigate this question, N- and L-*myc* expression was compared throughout murine development by coupling *in situ* hybridization to tissue sections and whole mounts of embryos with expression analysis using the polymerase chain reaction (in collaboration with R. DePinho, Albert Einstein University, New York). Unlike c-*myc*, which is generally expressed in proliferating cells, N- and L-*myc* are differentially expressed during development, particularly in the nervous system. In early embryos, N-*myc* is expressed in the developing brain, neural tube, posterior region of the somites, and visceral arches. By mid-gestation, it is expressed differentially in the central and peripheral nervous system, the sensory retina, the differentiating region of the lens, the epithelium of the gut and lung, and in the whisker follicles. L-*myc* is expressed in early embryos in the brain and neural tube, and in the CNS, lung and nasal epithelium in older embryos. N- and L-*myc* genes have been found to display both unique and shared expression patterns with respect to cell and tissue type. The studies of the *hox* and *myc* gene families raise the possibility that both differential and combinatorial expression of these genes may be important for normal development.

Recently, advances in mouse genetics, experimental embryology, and molecular biology have provided the unique opportunity for designing and creating defined mutations in the mouse. These new techniques allow the inactivation of one or both copies of a particular gene via homologous recombination with a defective gene. It has been estimated that in mammalian cells, targeting particular *loci* via homologous recombination occurs with sufficiently high frequency that cells with the desired genotype can be detected, isolated and cell lines established. Efficient selection schemes exist, which permit detection of rare integration events in the DNA. Such techniques can be readily applied to embryonic stem (ES) cells. These pluripotent cells, isolated from the mouse blastocyst, can be propagated in tissue culture for many passages, while still retaining the ability to contribute to the germ cell lineage after injection into recipient blastocysts. This provides a route for a particular genotype, isolated *in vitro*, to be transferred to a living animal and its progeny. Germ line transmission of an altered gene in the ES cell has already been achieved, and this promising method can therefore be used to introduce mutations in a variety of genes and to analyze their effects in the animal.

This type of targeted mutagenesis is being used to expand our understanding of the function of c-*mos* and c-*fos*, proto-oncogenes uniquely involved in mouse development. Specific mutations have been generated in these genes, and all the techniques have been established that should enable members of the Laboratory to introduce these mutations first into ES cells and later into the mouse germ line. If successful, this type of experiment will corroborate genetic evidence for the involvement of homeobox, *myc*, and other gene families in the regulation of mouse development. Future plans include the use of gene targeting by

homologous recombination to generate mouse models of human genetic diseases that are caused by single gene defects.

In another project, members of the Laboratory have taken advantage of the simple architecture of the eye lens, an epithelial tissue with a well studied development, to design a model system in which to examine differentiation and its relationship to neoplasia in the living mammalian organism. In the first of two independent studies, the ability of polyoma virus large T antigen (PyLT), an immortalizing oncogene, to promote lens transformation was tested. Two lineages of transgenic mice were created, which carry a gene fusion of the murine  $\alpha$ -A-crystallin promoter to the coding sequence of PyLT. A dominant, heritable trait of microphthalmia was observed in these transgenic lines. Histological analysis of lenses from day 13.5 of embryonic development through adult life revealed failures in cell elongation, denucleation, and mitotic senescence, indicating that the normal program of primary and secondary fiber cell differentiation is impaired. Immunohistochemical and biochemical analyses correlated the presence of PyLT with the observed abnormalities in lens development. Unlike other instances of immortalizing oncogene action in transgenic mice, no evidence for hyperplasia or tumor formation can be detected in these animals. Cells established *in vitro* from PyLT lenses continue to produce PyLT and crystallins and, different from their normal counterparts, proliferate continuously in culture. Thus, *in vitro*, developmentally perturbed lens cells show high proliferative capacity, whereas *in vivo* they do not. Present efforts are directed toward assessing the proliferative capacity of PyLT lens cells under various *in vivo* and *in vitro* growth conditions.

In the second study, the relationship of neoplastic growth and tissue differentiation in the eye lens were examined. Again the regulatory region of the mouse  $\alpha$ -A-crystallin gene was used, this time to target SV40 tumor antigens, strong tumor producing agents, to the eye lens in transgenic mice. SV40 oncogene products begin to accumulate at mid-gestation, and the animals develop lens tumors, which eventually invade neighboring eye tissues. More recently, the initiation of expression of SV40 tumor antigens in the embryonic lens and its relationship to tumor progression have been scrutinized. Two lines of transgenic mice with distinct tumor phenotypes have been studied in detail. The  $\alpha$ -T1 line develops fast growing, poorly differentiated lens tumors, whereas the lens tumors produced in the  $\alpha$ -T2 line are slow growing and well differentiated. There is a striking difference between these two lines in the temporal and spatial patterns of tumor antigen expression during initial lens development. In the  $\alpha$ -T1 line, the transgene is expressed very early in development in most lens cells, and no primary fiber differentiation takes place. In the  $\alpha$ -T2 line, transgene expression occurs after primary fiber formation has been initiated, and is restricted to differentiating fiber cells. In both mouse lines, levels of plasminogen activators, factors implicated in invasive growth, reflect the degree of malignancy found in the lens tumors. The anterior epithelial cells from both  $\alpha$ -T lines undergo normal development and remain morphologically normal until after birth, although in  $\alpha$ -T1 mice, they produce considerable amounts of SV40 tumor antigens. This suggests that the state of differentiation of the lens cell plays a critical role in its response to oncogene products. In collaboration with Dr. B. Sauer (duPont and Nemours, Wilmington), experiments are in progress to utilize the ability of the bacterial enzyme CRE to synapse DNA at LOX sites, for the purpose of modulating the timing and quantity of transgene expression in the mouse. With the help of this experimental approach, it is hoped that the postulate can be tested that both the timing of expression of an oncogene and the dose of the oncogene product in a differentiating tissue are of crucial importance in determining the extent of subsequent growth deregulation. The question also being posed is whether induction of differentiation in the anterior epithelial cells of  $\alpha$ -T1 or  $\alpha$ -T2 lenses will suffice to elicit neoplastic growth. If verified, this result would underscore the importance for oncogenesis of factors that define the differentiated state of a cell or tissue *in vivo*.

Cells established *in vitro* from  $\alpha$ -T2 lenses continue to produce all major forms of lens crystallins. A cDNA clone encoding a protein that binds specifically to a transcription element of the  $\alpha$ A-crystallin gene has been obtained from these cells. This putative lens

transcription factor is presently being characterized in collaboration with the laboratories of Drs. Piatigorsky (NEI) and Ozato (LDMI).

In a different collaborative project with Dr P. Furth (NCI), analysis of transgenic mice that express AIDS virus promoters fused to a marker gene was continued. The placenta was identified as an organ that contains more marker product per unit weight of total protein than any other cell system tested. This finding is of obvious importance for assessing the risk of children born to AIDS virus infected mothers.

Several collaborative studies are underway to take advantage of the technology, largely developed by members of this Laboratory, of directing the expression of clinically useful proteins to the mammary gland of transgenic animals. In collaboration with Dr. L. Hennighausen (NIDDK), the possibilities are being explored of expressing large amounts of proteins that are needed for the treatment of AIDS and other diseases, in the milk of transgenic animals. Last year, the group described their findings that human tissue plasminogen activator (tPA) and CD4 proteins can be produced in the milk of transgenic mice. These mice carry regulatory sequences of the mouse whey acidic protein (WAP) gene fused to tPA and CD4 coding sequences. In an extended collaboration with Dr. R. Wall (USDA, Beltsville, MD), the WAP gene has been inserted into the germ line of pigs. It should eventually be possible to produce in the milk of these and other species of farm animals therapeutic proteins in quantities that are sufficient for industrial realization.

## **LABORATORY OF MAMMALIAN GENES AND DEVELOPMENT**

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## THE LABORATORY OF DEVELOPMENTAL PHARMACOLOGY

**Daniel W. Nebert, M.D., Chief**



The Laboratory studies the molecular biology of genes encoding drug-metabolizing enzymes. The clinical discipline involving the study of genetic differences in drug metabolism has been termed pharmacogenetics. Steroids, fatty acids, prostaglandins, leukotrienes, biogenic amines, pheromones, and plant metabolites are metabolized by the P450 enzymes. These enzymes also metabolize innumerable chemicals in foodstuff, as well as drugs, chemical carcinogens and mutagens and other environmental contaminants. The large degree of overlapping substrate specificities, classes of inducing agents, and drug interactions have caused great difficulty in P450 studies at the level of catalytic activities and protein immunochemistry. P450 enzymes represent the classical "Phase I" metabolism in which the substrate is oxygenated. "Phase II" enzymes often use the oxygen as a site for further metabolism (e.g., quinone reduction, glucuronidation, and sulfate, glutathione, or glycine conjugation). Detoxification usually requires both Phase I and Phase II enzymes. Ironically, the intermediate formed after Phase I metabolism is often more toxic, carcinogenic or mutagenic than the original Phase I substrate.

Hundreds of drugs and other chemicals are known to stimulate (induce) their own metabolism or the metabolism of structurally related compounds. In addition, steroids, prostaglandins, and small peptide hormones have been found to regulate some of these activities. The mechanisms surrounding the induction of these enzymes and expression of these genes are of central importance to such fields as molecular genetics, developmental biology, teratogenesis, chemical carcinogenesis and mutagenesis, endocrinology, limology, drug addiction and tolerance, and toxicity.

The Section on Pharmacogenetics, under the direction of **Daniel Nebert**, is interested in the regulation and expression of genes encoding Phase I and Phase II drug-metabolizing enzymes. Work in this Section is divided into four areas: P450 gene nomenclature and evolution, P450 gene regulation, oxidative stress, and clinical applications in the field of pharmacogenetics. Experimental systems include recombinant DNA technology, inbred mouse strains, transgenic mice, somatic cell genetics, mutant cell lines in culture, and clinical material.

Most of the Phase I enzymes represent P450 proteins. The P450 gene superfamily is presently known to comprise sixteen P450 gene families, nine of which exist in all mammals. The P450 gene superfamily is ancient and has expanded via divergent evolution. The ancestral P450 gene was present probably more than two and a half billion years ago. Estimates of the unit evolutionary period (UEP; millions of years required for 1% divergence in amino acid sequence) range between 4 and 14, indicating a striking nonlinearity for the P450 superfamily. Four major factors might contribute to this nonlinearity: (i) archaeological evidence suggests that evolution is not uniform; (ii) certain amino acids or regions of the P450 protein might need to be retained, or conserved, for the enzyme to carry out the catalytic function required for survival advantage to the organism; (iii) "molecular drive," a non-Mendelian mechanism operationally distinct from natural selection and genetic drift, might aid the organism in spreading new variant genes in response to severe selective pressures, such as dietary changes; and (iv) gene conversion events can reestablish 100% similarity in a portion (or all) of a gene that had been considerably diverged from its homologous neighbor located nearby on the same chromosome. To improve our understanding of the evolution of the P450 superfamily, this Laboratory has recently isolated and sequenced the first trout and insect P450 genes.

Another focus of the Laboratory's interest is the regulation of P450 genes. At present, regulation of *CYP1A1* (trivial name P<sub>450</sub>) gene expression has been more extensively characterized than that of all other P450 genes combined. This Laboratory has pioneered such regulatory studies through use of the mouse hepatoma Hepa-1 wild-type cultures and receptor-defective and *Cyp1a1* metabolism-deficient mutant cell lines. These lines have been used for transfecting the reporter gene chloramphenicol acetyltransferase (CAT gene) driven by various upstream regions of the *Cyp1a1* gene. It can thus be determined which upstream regions require a functional aromatic hydrocarbon (Ah) receptor and which regions require *Cyp1a1* metabolism. Upstream *Cyp1a1* regulatory elements include: (i) the TATA box; (ii) a proximal element between -245 and -150, which is absolutely essential for constitutive and inducible gene expression; (iii) a distal element, i.e., the tetrachlorodibenzo-p-dioxin (TCDD)-inducible enhancer between bp -1100 and -880; and (iv) an additional element that participates in a negative autoregulatory loop. Both the TCDD-inducible enhancer and the negative autoregulatory loop appear to require a functional Ah receptor. The transcriptional preinitiation complex that up-regulates these genes is believed to include the Ah receptor (bound to endogenous or foreign ligand) and at least one other protein, which confers chromatin binding properties. Site-directed mutagenesis of these elements, *in vivo* genomic footprinting, competition studies with gel mobility shift assays, and studies on phosphorylation requirements are underway for the *CYP1A1* gene in mouse, human and trout, as well as for the mammalian *CYP1A2* gene (trivial name P<sub>450</sub>).

This year, the study of the control of a gene for NAD(P):menadione oxidoreductase has shed some light on how organisms deal with oxidative stress. The gene is part of the [*Ah*] gene battery, which comprises six genes encoding enzymes with coordinate metabolic functions—*Cyp1a1*, *Cyp1a2*, NAD(P)H:menadione oxidoreductase (*Nmo-1*), aldehyde dehydrogenase (*Aldh-1*), UDP glucuronosyltransferase (*Ugt-1*), and glutathione transferase (*Gt-1*). Metabolism of endogenous substrate(s) by the *Cyp1a1* gene product not only appears to control its own constitutive expression but also the expression of the other five genes, and all six are under the coordinate control of the Ah receptor. All six of these TCDD-inducible genes have been cloned. Using *14CoS/14CoS* radiation deletion homozygote mice, a gene (*Nmo-1n*) that encodes a negative effector of *Nmo-1* (located on mouse chromosome 8) has been localized on mouse chromosome 7. The *Nmo-1* enzyme plays a major role in protecting animals from "oxidative stress," such as the ingestion of quinones and other phytoalexins present in foodstuff. In collaboration with Dr. Albert Fornace, (NCI), three growth arrest-inducible and DNA damage-inducible (*gadd*) genes have been found, which are also activated in these *14CoS/14CoS* radiation deletion homozygote mice, suggesting that the *gadd* genes are also coordinately regulated by a chromosome 7 gene encoding a negative effector. Whether *Nmo-1* and the *gadd* genes are regulated negatively by the same gene on mouse chromosome 7 remains to be determined. This region of mouse chromosome 7, containing one or more genes encoding negative *trans*-acting factors, appears to resemble the

*recA/lexA*-controlled SOS response in prokaryotes. Work is in progress to clone and characterize the *Nmo-1n* gene and other genes encoding *trans*-acting factors.

A long-term goal of this Laboratory is to investigate the hypothesis that polymorphisms of the enzymes of the P450 family have clinical relevance to an individual's risk for cancer. Thus, members of the Section have cloned and sequenced the human *CYP1A1* and *CYP1A2* genes and their flanking regions, and have localized both genes near the *MPI* locus on chromosome 15. In the human population, they have shown at least 15-fold variations in lymphocyte *CYP1A1* inducibility and in hepatic constitutive *CYP1A2* gene expression. Evidence suggests that the human *CYP1A1* and *CYP1A2* genes are important in the metabolic formation of chemical carcinogens, mutagens and teratogens, as are the orthologous genes in laboratory animals. Mendelian inheritance of restriction fragment length polymorphisms (RFLPs) of the *CYP1A1* gene has been determined by this Laboratory, and families with high and low cancer incidence are being studied. This Laboratory has also cloned and characterized the human *NMO1* gene, and it is predicted that its activity will also vary in the human population. In the future, it should be possible to correlate RFLP patterns of these genes with human disease. Such tests would facilitate the evaluation of cancer and toxicity risk for individuals exposed to foreign chemicals. These assays would aid the individual, employer and physician in decisions regarding life style, cigarette smoking, employment, and prescription drugs.

The Unit on Microbiology, under the direction of Alvaro Puga, studies various molecular mechanisms involved in *CYP1A1* gene regulation. This group uses a variety of approaches to clone the Ah receptor gene, one of several transcription factors involved in *CYP1A1* expression, and responsible for induction of the gene by certain foreign chemicals. Two new regulatory pathways have been uncovered during the study of murine *Cyp1a1* expression by this group. The first involves the enzyme poly(ADP)ribose polymerase. With the recognition site probe binding assay, a recombinant cDNA clone encoding this enzyme was isolated and shown to bind to the upstream regulatory elements of the *Cyp1a1* gene. The involvement of this enzyme in *Cyp1a1* gene transcription was demonstrated by using an inhibitor of poly(ADP-ribose) polymerase, which was found to repress mRNA synthesis. The second regulatory pathway was found in experiments with *Cyp1a1* deficient ( $P_1^-$ ) mutant cell lines. These cells have very high, deregulated levels of *Cyp1a1* mRNA synthesis and no functional *Cyp1a1* enzyme, due to mutations in the structural gene. Introduction of a functional *Cyp1a1* gene, or, surprisingly, the human *CYP1A2* gene, into these cells restores the regulation of the endogenous *Cyp1a1* gene to its constitutive levels of expression. It is clear that an auto-regulatory feedback loop acts as a major component in *Cyp1a1* transcription and that this regulatory mechanism involves the oxidation of an as yet unknown substrate.

## LABORATORY OF DEVELOPMENTAL PHARMACOLOGY

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## LABORATORY OF DEVELOPMENTAL NEUROBIOLOGY

Phillip G. Nelson, Ph.D., Chief



Left to right: D. Brenneman, D. Klein, A. Buonanno, Y-P. Loh, E. Neale, M. Mayer, J. Russell, P. Nelson

The Laboratory is broadly concerned with cellular and molecular mechanisms of nervous system development. In pursuing this theme, the Section on Neurobiology, led by **Phillip Nelson**, has investigated the "sculpturing" effects of neural activity patterns imposed by structured stimulation from the environment during development, which is critical for the functional architecture of the nervous system. Considerable progress has been made by Nelson, Fields, Yu and Neale in the last year in analyzing the activity-dependent synapse stabilization and elimination involved in this sculpturing. In the three-compartmental tissue culture system used by these workers, a plasticity phenomenon has been demonstrated, whereby chronic stimulation (for 4-5 days) of sensory afferents to spinal cord (SC) neurons confers a synaptic advantage on the stimulated afferents relative to unstimulated afferents converging onto the same SC neurons. They have shown that specific blockade of one type of excitatory synaptic receptor, the NMDA receptor, on the SC neurons by the potent NMDA antagonist, APV (also known as AP5), prevents this response to chronic stimulation. The plasticity, however, is not exclusively related to NMDA receptor activation, because the plasticity can be detected under conditions of NMDA receptor blockade, if the concentration of calcium ions in the culture medium is raised. They hypothesize that free intracellular calcium ion concentration is critical in regulating both synapse-augmenting and synapse-eliminating cell biologic processes. The NMDA receptor is only one mechanism, albeit a potent one, involved in activity-dependent changes in intracellular calcium. Collaboration has begun with Dr. Russell in the unit on Neuronal Secretory Systems, with the aim of making direct measurement of this latter critical variable in living neurons and glial cells. Pharmacological manipulations to test hypotheses as to second messenger involvement

in synapse development and effects of protease inhibitors on synapse elimination, are contemplated.

Although ongoing electrical activity appears to exert a positive effect on synapse stabilization, a detailed analysis of chronic phasic stimulation and neurite outgrowth indicated very little effect. However, the same stimulation induces an acute inhibition of neurite growth and a change in the morphology of the growth cone toward a more primitive form. The morphological substrate for the growth cone alteration, as well as the role of calcium in producing these acute changes and in the eventual recovery, will be investigated. It remains possible that electrical activity at a critical interval during synaptogenesis may act to stabilize extant connections and inhibit further growth of neurites that have not yet established a synapse.

The major focus of the Unit on Neurochemistry, led by **Douglas Brenneman**, is cellular communication, which regulates epigenetic development of central nervous system. The molecular basis of such communication is undoubtedly complex, but this group has pioneered the idea that neuropeptides mediate communication between neurons and non-neuronal cells. The results of these interactions have profound consequences for the shaping and composition of neuronal networks in the CNS. Progress in characterizing the significance of one particular peptide, vasoactive intestinal peptide (VIP), has been hampered by the lack of specific drug antagonists, which could be used to delineate its function both *in vitro* and *in vivo*. A hybrid peptide drug has been synthesized, and found to have potent VIP antagonistic properties in such diverse assays as: sexual activity in rats, VIP-stimulated adenylate cyclase, VIP-mediated protein secretion from astroglia, VIP radioligand binding, and neuronal survival in developing CNS cultures. In addition to providing much needed pharmacological agents for the study of neuropeptide function, this group has also studied the ontogeny of mRNA for various neuropeptides found in CNS cultures. Of particular importance is the discovery that large amounts of mRNA for VIP are present during a developmental period when rapid neuronal growth and differentiation occurs. The temporal expression of the VIP gene and the neurotrophic aspects of exogenous VIP (determined in previous studies by this group) combine to make a strong case for the biological significance of this peptide during development. The complexity of the consequences of VIP's interaction with non-neuronal cells has begun to emerge in their studies of secreted proteins, elicited by sub-nanomolar concentrations of the peptide. It is now apparent that VIP releases many proteins from cortical astrocytes, some of which increase the survival of developing neurons. The characterization of these glial-derived proteins will be a major focus of future work for this group.

In parallel, but complementary, studies this Unit continues to investigate the possibility that the hippocampal neuronal death produced by gp120 (the external envelope protein of the Human Immunodeficiency Virus) is caused by interference with endogenous neurotrophic substances, perhaps VIP. Recent work has suggested that gp120 does not act directly on hippocampal neurons, rather it interacts with non-neuronal cells to produce neuronal cell death. In addition, a neuronal survival assay has been used to detect neurotoxic material present in the cerebrospinal fluid from patients with AIDS dementia. All the toxicity associated with the CSF of these patients has been shown to be attenuated by the drug Peptide T, and by monoclonal antibodies to murine CD4, thus suggesting the specificity and possible prevention of these deleterious effects. In summary, this group is committed to the further exploration of neuropeptide-related mechanisms important to neurodevelopment, and the application of these findings to human disease.

Tetanus toxin has been studied for several years by the Unit on Cell Biology, under the direction of **Elaine Neale**, both in terms of its physiological effect on neurons and because of its usefulness as a marker for the neuronal surface membrane. These studies have been re-initiated, coincident with other developments in the Laboratory. The patch clamp technique allows the recording of electrophysiological events in very small cells, and thus it has become possible to follow the electrical development of newly cultured neurons. The Unit has demonstrated that tetanus toxin binds to the surface of such young neurons and can

be used with immunohistochemistry to stain neurons in living (i.e., non-fixed) cultures. It was demonstrated further that Fragment C, the binding portion of the toxin molecule, labels young neurons equally well, with the added advantage that it does not appear to interfere with electrophysiology. These results, together with the acquisition of a high sensitivity video camera and optical memory disk recorder, offer the possibility of following the morphological development of freshly plated cells. Such studies, coupled with image analysis, should provide information on neuronal complexity and on events leading to the formation of contacts with target cells. The ability to obtain correlative electrophysiological analysis provides a functional component to the study.

The use of Fragment C as a neuronal stain opens questions about the kinetics of its binding to neurons. Other cell biological issues arise, concerning differences in the cellular processing of holotoxin and its binding and active fragments, and differences in the processing between young and more developed neurons. Preliminary studies have indicated that developmental state is related to the severity of neurotoxicity, similar to the finding for tetrodotoxin. Central to all of these issues is the mechanism whereby the toxin blocks synaptic activity. Using radiolabeled toxin, Fragment C, and various characterized monoclonal antibodies, data on some of the kinetic issues has been obtained. Bound Fragment C can dissociate from the neuronal surface and retain its ability to re-bind; tetanus toxin's association with the neuron is more stable, and it is internalized more readily. A specific monoclonal antibody stabilizes the association of Fragment C, and has very little effect on holotoxin. Gel electrophoresis has provided a time course of toxin breakdown, and indicates that the toxin is degraded in or on neurons, and that breakdown products are released into the medium. Using these biochemical data as a basis, morphologic studies involving immuno-electron microscopy will be undertaken. The fine structural localization of toxin will be correlated with its electrophysiological effects, and may help in understanding how the toxin acts at the synapse and how some neurons recover function.

The Unit on Molecular Neurobiology, led by Andres Buonanno, concerns itself with the molecular biological analysis of the effects of the neurotransmitters on the postsynaptic cell, which vary according to the type of receptors expressed. The two major types of receptors, located on postsynaptic targets, are either linked to G proteins or activated directly by binding of the ligand. The latter, known as ligand-gated channels, open an ion selective pore when bound to a specific neurotransmitter. Regulating the temporal expression, location, and availability of functional receptors on the cell surface can modulate interneural communication, which could consequently affect fundamental processes, such as cognition, learning and memory. A major interest of the Unit is how the expression and function of ligand-gated channels are modulated during development and by neural activity. Studies have focussed on two types of ligand-gated channels; the nicotinic acetylcholine receptors (nAChRs) and the excitatory amino acid receptors (EAARs).

Although different ligand-gated ion channels bind different neurotransmitters, and are selectively permeable to ions of different charge and radius, it was recently discovered that they are evolutionarily related. The subunits of nAChRs, GABA and glycine receptors, which are members of this gene superfamily, have four conserved putative transmembrane spanning regions, thought to line the ion pore, and an extracellular domain flanked by disulfide-bonded cysteines, thought to form part of the agonist binding site. EAARs are also believed to be members of the ligand-gated channel gene superfamily. Therefore, Dr. Raluca Eftemie and Dr. Andres Buonanno used degenerate oligonucleotide probes, coding for conserved sequences extending the intracysteine domain, to screen cDNA libraries for EAAR clones. In theory, the degenerate oligonucleotide mixture should hybridize to all members of the gene superfamily. To maximize the chances of identifying clones coding for EAARs, lobster muscle and *Drosophila* cDNA libraries were screened (invertebrates use glutaminergic transmission at their neuromuscular synapse; thus invertebrate muscle represents an enriched source of EAAR mRNA). A 1.7 kb *Drosophila* cDNA clone was partially sequenced, and shown to have homology to members of the ligand-gated ion channel family in the intracysteine domain; other conserved sequences in the transmembrane domain have not yet

been identified. Twenty five genomic clones hybridized to the probe; these are presently being subcloned, sequenced, and characterized.

A *Xenopus* oocyte expression system was established in the laboratory by Dr. Buonanno to functionally identify the type of receptors coded by the cDNAs cloned. This technique consists of injecting *in vitro* synthesized RNA, made from the cDNA clones, into oocytes, and using two-electrode voltage clamp to measure neurotransmitter-induced currents. The system was initially optimized by injecting rat brain poly-A RNA, and inducing depolarizing currents by the application of glutamate, kainate and quisqualate (glutamic acid analogs). The responses are qualitatively and quantitatively similar to those published by other groups. Transmitter-induced currents are measured in oocytes injected with RNA synthesized from large pools of cDNA clones that hybridized to degenerate oligonucleotides coding for the intracysteine domain. The first batch of fifty lobster muscle cDNA clones failed to elicit responses when glutamate, quisqualate or kainate were applied to the bath. This approach, which is a modification of the "sib selection" procedure initially used to clone serotonin and substance K receptors, will be further used to screen lobster muscle and rat brain cDNA libraries for kainate and quisqualate receptors.

Historically, the neuromuscular junction has served as a model for understanding synaptogenesis and synaptic function. In mammals, synaptic transmission at the neuromuscular junction is cholinergic, and mediated by nAChRs. Although this ligand-gated channel has been studied extensively at the biochemical level, little is known about the mechanisms that regulate its transcription during development and by neurally-induced electrical activity. In collaboration with Drs. John Merlie and Michael Crowder (Washington University, St. Louis), Dr. Buonanno showed that a 750 bp upstream region of the mouse nAChR gamma subunit gene confers proper tissue-specific and developmental regulation, when transfected into mouse C2 skeletal muscle cells. This work is being extended by Dr. Laura Lautens, who is delineating the enhancer sequences by creating serial deletions in the 750 bp upstream sequence, using the polymerase chain reaction procedure, inserting the constructs in chloramphenicol acetyltransferase (CAT) expression vectors, and quantitating CAT activity in transfected muscle cells. In collaboration with Dr. Nelson, experiments have been started to study the down-regulation of nAChR expression by electrical activity. Jonathan Smith is optimizing conditions to stimulate cultures of C2C12 skeletal muscle cells by applying field currents, and testing for the effect of these currents on nAChR gene transcription. Using this approach, constructs containing transcription regulatory elements in CAT expression vectors will be tested to identify the *cis*-acting sequences that respond to electrical activity.

The Unit on Neurophysiology and Biophysics, led by Mark Mayer, has continued to explore the physiology, biophysics and cell biology of excitatory amino acid receptors. Experiments by Drs. Vyklicky, Patneau and Mayer use a fast perfusion system for rapidly applying excitatory amino acids. This is allowing analysis of dose response curves for activation of N-methyl-D-aspartate (NMDA) receptors on hippocampal neurons by transmitter candidates, such as L-glutamate and L-homocysteate, as well as providing valuable insight into structure/activity relationships for agonist action at NMDA and non-NMDA receptors. L-glutamate is the most potent NMDA receptor agonist found in the brain ( $K_d = 2.2 \mu M$ ), followed by L-homocysteate ( $12 \mu M$ ) and L-aspartate ( $17 \mu M$ ). Similar experiments are in progress with a family of sulfur amino acids, errors in the metabolism of which lead to severe neurological deficits in man. Due to the fast onset of desensitization and the neurotoxic action of NMDA receptor agonists, such experiments are difficult to perform and interpret, without the fast perfusion system. The high potency of endogenous NMDA receptor agonists raise fundamental questions about the mechanisms that regulate the concentration of these potentially neurotoxic transmitter substances in the extraneuronal environment.

Similar experiments by Vyklicky, Benveniste (NINDS) and Mayer are under way to determine the mechanisms by which glycine regulates desensitization at NMDA receptors, and are providing important new contributions to receptor theory. A major finding was that glycine reduces NMDA-evoked desensitization by increasing the rate of recovery from

desensitization. Quantitative analysis of the potentiation of NMDA receptor activity, as a function of glycine concentration, showed differential effects on peak and steady state responses, which can be explained by a four-fold higher affinity for glycine at the peak of NMDA response, compared with the steady state response. This suggests that binding of NMDA produces a conformational change in the receptor, which lowers the affinity for glycine. "Desensitization" thus reflects dissociation of glycine from NMDA receptors. A computer model of NMDA receptor activity has been developed by John Clements (NINDS) to help explore this hypothesis. The model implies that the probability of activation of NMDA receptor channels is close to zero in the absence of glycine. Experiments by Benveniste and Mayer using the glycine antagonist 7Cl-kynurenic acid confirm this, since it is possible to completely block activation of NMDA receptors with a dose of antagonist that is entirely selective for the glycine binding site.

Experiments by Benveniste, Mienville, Sernagor and Mayer have used concentration jumps with antagonists to explore structural features required for block of NMDA receptor activity by ligands that act competitively at the NMDA and glycine binding sites. The most interesting finding to date concerns the NMDA antagonists, CPP and AP5, which are structurally related to glutamate, and which have similar equilibrium affinities, but markedly different association and dissociation rates. In CPP the glutamate backbone is conformationally restricted, as part of a piperazine ring, while AP5 is a linear molecule. Future experiments with other antagonists are planned, as these compounds are developed by the pharmaceutical industry, and could provide molecular insight into features required for activity as NMDA antagonists.

The Section on Neuroendocrinology, directed by David Klein, has made a series of discoveries of basic importance in signal transduction and the molecular biology of the pineal gland, which functions as part of a system that measures the duration of the night period. The pineal gland converts this measure into a hormonal signal—elevated levels of melatonin at night. In addition, these findings have significance in the field of transsynaptic regulation of neuronal metabolism in general.

Several advances were made this past year in the area of signal transduction. Olga Nikodijevic discovered that there are receptors on the pinealocyte for adenosine, which regulate cyclic AMP and cyclic GMP production. It was also found that adenosine is formed from extracellular ATP by an enzyme system, which appears to be located on the external surface of pineal cells. This is important in signal transduction, because ATP is released along with norepinephrine and other transmitters, which are stored with ATP in a complex. Accordingly, these findings point to adenosine as a neural transmitter in the pineal gland.

Li Yu investigated phosphorylation of pineal proteins, in particular to identify substrates of cyclic GMP dependent protein kinase. To accomplish this, he purified this enzyme from brain, and found that incubation of cyclic GMP, purified cyclic GMP dependent protein kinase, and pineal supernatant fractions results in phosphorylation of two proteins. This is the first clear evidence of any effect of cyclic GMP on pineal metabolism.

Juan Antonio Reig and Li Yu have studied substrates of cyclic AMP dependent protein kinase in the pineal gland. This effort has revealed the presence of a 33 kDa phosphoprotein, which is phosphorylated in intact cells by an adrenergic/cyclic AMP dependent mechanism. This protein is of further interest in signal transduction, because it appears to exist in a complex with the  $\beta\gamma$  subunit of GTP binding regulatory protein. A similar situation exists in the retina, making these tissues striking exceptions to the generalization that the  $\beta\gamma$  subunit is limited to the membrane. Hence, it is now clear that this regulatory system probably has cytoplasmic function. This team has also identified the  $\alpha_i$  subunit of the inhibitory GTP binding regulatory protein in pineal cytoplasm. It seems that these elements participate in a system that modifies signal transduction.

Anthony Ho and Connie Chik have discovered that norepinephrine increases the intracellular pH of the pinealocyte, and that this is accomplished by activation of an  $\alpha_1$ -adrenergic

→[Ca<sup>2+</sup>]<sub>i</sub>→ protein kinase C mechanism. This change in pH may be of special importance in the regulation of the cyclic GMP response to norepinephrine, because when this change is blocked, the cyclic GMP response is markedly reduced. Another possible effect of pH may involve protein/protein interactions. It is known that the 33 kDa/βγ complex described above dissociates at elevated pH.

Important advances in molecular biology of the pineal gland have been made by the Section, both with regard to the genes for signal transduction proteins, and for those of melatonin forming enzymes. Melatonin is formed from serotonin by two enzymes. The first is N-acetyltransferase; Joan Weller isolated putative N-acetyltransferase clones, using antiserum against sheep N-acetyltransferase, raised in a collaborative effort with Georgetown University Staff. These clones have been sequenced by Patrick Roseboom, and are at present being further characterized. The second enzyme in melatonin synthesis from serotonin is hydroxyindole-O-methyltransferase; Helena Illnerova cloned the human hydroxyindole-O-methyltransferase gene, which has now been sequenced by Susan Donohue. Future plans involve identification of the regulatory elements that cause the expression of this gene in the pineal gland and retina. Serotonin is formed from tryptophan in the pineal gland by two enzymes, the first of which has been studied by the Section. Joan Weller has cloned the gene for sheep tryptophan hydroxylase, which has been partially sequenced in a collaborative effort with NIMH staff. The photoneural regulation of the expression of mRNA coding for tryptophan hydroxylase is being studied, in part with cDNA probes. Finally, in a collaborative effort involving several laboratory members and members of the NEI, rat pineal S-antigen has been cloned and sequenced. This protein is found only in the pineal gland and in the retina, and appears to be involved in signal transduction.

The nerve terminal is a highly specialized region of a neuron, which is separated from the neuronal soma by an axon, and whose function is to release neurotransmitter quanta and to regulate the number of quanta secreted. Modulation of the quantity of the transmitter released at the terminal may form the basis for all central nervous system functions, including integration of information, and long term information storage and retrieval. This modulation is achieved by transduction of information content in the action potential train, and by local influences at the nerve terminal, via activation of receptors at the terminal and the consequent modification of the responses of the terminal membrane. Because of the complexity (cellular heterogeneity and complex organization) and extremely small size of the nerve terminal, basic understanding of the molecular mechanisms involved in their function in the central nervous system is lacking. The program of the Unit on Neuronal Secretory Systems, under the direction of **James Russell**, is focused on studying the biochemistry and physiology of the nerve terminal, using the neurohypophysial neuroendocrine cells as the model system. The nerve terminals of the neurons of the hypothalamo-neurohypophysial system, which secrete vasopressin or oxytocin, are discretely localized in the neurohypophysis, where they are accessible to experimental manipulations, both *in vivo* and *in vitro*. These nerve terminals can be isolated from the neurohypophyses without contamination by the postsynaptic membrane, unlike nerve terminals from other regions in the central nervous system. Furthermore, the posterior pituitary contains nerve terminals from only two very discrete types of neurohypophysial magnocellular neurons.

Studies on the functional organization of the nerve terminal form the central theme of this Unit. Currently, the Unit is investigating the importance of ionic channels and receptors to the initiation and modulation of secretion at the nerve terminal. Three major approaches are being employed: (1) The kinetics and magnitude of secretion from isolated nerve endings (neurosecretosomes) are studied. Agonists and antagonists of ion channels, receptors and enzymes are employed to investigate both the mechanism and regulation of secretion, using this model. (2) Toxins and other biochemical methods are used to investigate the molecular mechanisms of calcium-induced exocytosis. (3) Intracellular messengers (ion concentration measurements, and measurement of second messengers, such as cyclic GMP) involved in mediation, and modulation of secretion are measured.

The neurosecretosome preparation (isolated neuroendocrine nerve endings) has been maintained in culture for over 4 days. During this time, these nerve endings respond to depolarizing stimuli with calcium-dependent secretion of both vasopressin and oxytocin. They also respond to activation of receptors by modulation of hormone secretion. These cultured nerve endings are being used to study the dynamics of hormone secretion, and its modulation by receptor occupation. They are also used to identify ionic channels on nerve terminals, and their modulation by neuropeptide receptors on the nerve terminals, using state-of-the-art biophysical techniques.

The neurosecretosome preparation was also used to study the kinetics of secretion in response to depolarizing stimuli, with very high temporal resolution. These studies revealed that, during prolonged depolarizations, secretion undergoes inactivation. Dr. Kemal Payza has found that inactivation depends on extracellular calcium ions, and is not caused by the membrane potential change alone. Exposure to calcium ionophores, which cause secretion by elevating intracellular calcium, paradoxically also causes partial inactivation of secretion in the absence of membrane depolarization. Dr Payza has shown that calcium entry by itself is a necessary and sufficient stimulus for activation of secretion. Calcium is also necessary for the inactivation of secretion. Membrane depolarization *per se* does not seem to play a role in activation or inactivation of secretion, other than to open voltage gated calcium channels, which allows for calcium entry into the terminals. He has also used the nerve terminal preparation to identify intracellular second messengers involved in the modulation of somatostatin on secretion. The effects of neuropeptides, such as kappa opiates and somatostatin on secretion and their receptor-effector coupling are being investigated.

The neurosecretosome preparation provides an ideal model to study intracellular reactions involved in triggering and regulating neurosecretion. The use of toxins that block secretion has been shown to provide a means of identifying cellular substrates important in the exocytosis machinery. In collaboration with Dr. Jane Halpern (CBER, FDA), tetanus toxin at nanomolar concentrations was shown to completely block secretion induced by either depolarization or calcium ionophore. This inhibition depends on toxin internalization, and is blocked by tetanus antitoxin. The intracellular substrate of the toxin responsible for blockade of exocytosis is now being sought.

The high resolution video imaging microscope adds a new dimension to the investigation of the functional organization of the nerve terminal. This instrument should prove valuable in resolving long standing questions on the kinetics of calcium concentration increase in the terminal, and its homeostasis. Preliminary experiments show that calcium entry into the neurosecretosomes mediated by depolarization occurs mainly at one pole of the nerve endings, upon onset of the stimulus. Experiments are underway to correlate calcium and  $H^+$  ion transients with the rate of secretion. Furthermore, the mechanism by which opiate kappa-receptor activation brings about inhibition of secretion, the mechanism of tetanus toxin action, and the protein kinase C mediated increase in secretion will be studied in terms of the involvement of intracellular calcium concentration. Thus, quantitative light microscopy will be an important tool to study intracellular signals, using fluorescent probes.

The Section on Cellular Neurobiology, under the direction of Yoke Peng Loh, studies the enzymology and regulation of biosynthesis, packaging, secretion and function of the ACTH/endorphin  $\alpha$ -MSH family of neuropeptides. This family of peptides is involved in intercellular neurocommunication and neural development. The ACTH,  $\alpha$ -MSH and endorphin peptides are synthesized in the brain and pituitary from a common glycoprotein prohormone (pro-opiomelanocortin, POMC) of about 32,000 daltons in size. This group has continued to purify and characterize enzymes involved in the proteolytic processing of POMC. A prohormone converting enzyme (PCE) has been purified from bovine intermediate and neural lobe secretory vesicles and characterized as an acidic, aspartic protease, which cleaves POMC at specific paired basic residues to yield ACTH,  $\beta$ -endorphin and  $Lys\gamma_3$ MSH. PCE has been shown to be co-secreted with the POMC-derived peptides, in a regulated manner, from bovine intermediate lobe, confirming the presence of the enzyme in secretory vesicles. Dr. Nigel Birch has recently shown that PCE is a calcium-activated protease, and

that maximum stimulation of the enzyme was achieved with calcium concentrations in the 10–15 mM range. Such a range of free calcium concentrations has been reported in pituitary secretory vesicles. Furthermore, it was shown that the extent of POMC processing depends on the calcium concentration. Thus, differences in the intravesicular free calcium concentration can potentially determine the different degree of formation of some of the smaller POMC cleavage products (e.g.,  $\beta$ -endorphin, joining peptide) in the intermediate and anterior lobes of the pituitary.

Dr. Birch has also explored the possibility that 'O'-glycosylation of N-POMC may regulate the differential processing of this peptide in the two lobes of the pituitary. He incubated 'O'-glycosylated N-POMC, found primarily in anterior lobe, and the non-'O'-glycosylated N-POMC from the intermediate lobe with PCE and showed that the non-'O'-glycosylated form is cleaved to yield  $\gamma_3$ MSH 7–8 times faster than the 'O'-glycosylated form. The close proximity of the 'O'-glycosylated group to the cleavage site may result in steric hindrance to the enzyme, preventing efficient cleavage. His findings are the first demonstration that 'O'-glycosylation near a cleavage site can regulated the processing of a pro-protein, and can account for the processing of N-POMC to  $\gamma_3$ MSH in the intermediate lobe of the pituitary.

Dr. Loh has continued to investigate the signals involved in targeting POMC to the regulated pathway. Fusion genes of different POMC cDNAs, which had varying lengths of the 3' end deleted, were fused to the CAT gene. Each of these constructs was expressed in AtT-20 cells, using the *vaccinia* virus expression system. The CAT/POMC fusion proteins were shown to be expressed by AtT-20 cells, using the immunofluorescence method of staining for CAT. Immunofluorescence studies in cells expressing CAT fusion proteins revealed that 25 amino acids of the N-terminal POMC sequence are sufficient to route the prohormone into secretory vesicles. She also showed that forskolin treatment stimulates the secretion of CAT activity into the medium from stable transformants expressing CAT/POMC fusion protein with  $\geq 25$  amino acids of N-POMC. This regulated secretion of CAT activity confirmed the targeting of fusion proteins with  $\geq 25$  amino acids of N-POMC into secretory vesicles. Dr. Mogens Fenger has made several POMC/CAT constructs with mutations at some of these amino acids, and expressed them in AtT 20 cells. This line of research should reveal the critical amino acids necessary to preserve the targeting signal of POMC.

To understand the role of POMC-derived peptides in neural development, the temporal/spatial expression of POMC was examined by *in situ* hybridization and immunocytochemistry. Both POMC mRNA and immunoreactive POMC/ACTH are first observed in the presumptive arcuate nucleus of embryonic brain on gestational day 10–1/2 (E10–1/2). Immunostained fibers are also evident on E10–1/2 in the lateral and dorsal diencephalon. In the pituitary, POMC expression first appears in cells of the anterior lobe on E12–1/2 and in the intermediate lobe on E14–1/2. These findings reveal for the first time that POMC is expressed very early in development and, interestingly, is expressed in the CNS before the pituitary. Dr. Adrian Rius showed that the ACTH immunoreactivity seen at E10–1/2 is due to intact POMC, and that processing of the prohormones does not begin until E11–1/2. HPLC analysis of processed products at E11–1/2 revealed that des-acetyl  $\alpha$ -MSH and  $\beta$ -endorphin are the major POMC-derived peptides present. No  $\alpha$ -MSH was observed. In collaboration with Dr. C. Coscia (St. Louis University), Dr. Rius has shown that  $\mu$  receptors first appear in mouse embryos at E12–1/2, one day after POMC processing in the CNS has begun. The embryonic  $\mu$  receptor exhibits the same K<sub>d</sub> as in adult brain, but the maximum specific binding is different. Moreover, displacement studies with various ligands indicate that these early embryonic  $\mu$  receptors are specific for the POMC-derived peptide,  $\beta$ -endorphin. It appears, therefore, that as early as E12–1/2, the embryonic pattern of processing of POMC is already established, and the receptor for at least one of these POMC-derived peptides ( $\beta$ -endorphin) is present, indicating that the POMC system is fully functional at this stage to exert its effect on CNS development. The role of des-acetyl  $\alpha$ -MSH and  $\beta$ -endorphin in neuronal cell proliferation and neurite outgrowth is currently being investigated.

To address the issue of how the initiation of neuropeptide phenotypes is regulated in the vertebrate CNS, Dr. Hayes has continued to use *in situ* hybridization histochemistry to determine the temporal map of POMC mRNA expression in the embryonic frog CNS. POMC gene expression was first seen at developmental day 1.3 (Stage 28) in cells of the embryonic stomodeal-hypophyseal plate. By day 2.3 (Stage 37/38), they are found near the notochord, where they are presumed to form the anterior and intermediate lobes of the pituitary. It appears from these *in situ* studies that many pituitary precursor cells are already differentiated, as they pass in proximity to the developing brain. In the CNS, POMC mRNA labeling is first seen at Stage 31 in the neural tube in cells of the diencephalon. These cells appear to radiate from the point at which the hypophyseal plate makes its closest pass to the embryonic brain, raising the hypothesis that the onset of expression of this gene in brain depends on interactions with the hypophyseal cells.

To understand the regulation of expression of the POMC and pro-enkephalin genes during CNS development, Drs. May Wong and Judith Hewitt have cloned these two genes in the *Xenopus laevis*. Dr. Wong has now obtained 8 kb of 5' DNA sequences of the pro-enkephalin gene, and is currently identifying the putative regulatory sequences.

## LABORATORY OF DEVELOPMENTAL NEUROBIOLOGY

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## LABORATORY OF THEORETICAL AND PHYSICAL BIOLOGY

David Rodbard, M.D., Chief



Left to right: P. Munson, A. Chrambach, D. Rodbard, A. Yergey

The Laboratory of Theoretical and Physical Biology has continued a unique, highly productive multidisciplinary program involving theoretical, statistical, biochemical and physical-chemical approaches to problems relevant to child health and human development. The Section on Theoretical Biology, led by **David Rodbard**, is engaged in the development of computer programs to assist in the management of insulin-dependent diabetes mellitus. A minimally complex mathematical model was developed to describe the essential features of the pharmacokinetics and pharmacodynamics of subcutaneous insulin. This program allows one to predict the effects of changes in types, regimen, or dosage of insulin, and the timing and carbohydrate content of meals, and can be used for education of patients and physicians. After further studies to validate the model, this program will be available to make recommendations to physicians about patient management. Other programs have been developed and refined for analysis of data from self-monitoring of blood glucose. These programs utilize data entered automatically from glucose meters equipped with memory and clock-calendar, and show the circadian glucose profile in relation to the profile of insulin action.

Another focus of the Branch's work is to develop new algorithms and computer programs to assist in other endocrinological research, and laboratory studies of receptors for hormones, drugs, and neurotransmitters. The Unit on Biostatistical Methodology, under the direction of **Peter Munson**, has developed several new methods and algorithms. The non-parametric "FLEXIFIT" algorithm, a "universal," self-modelling approach to describe families of dose response curves, has been revised to improve efficiency and speed of computation and to estimate the effective number of degrees of freedom, the root mean square error, standard errors of parameters, and to improve hypothesis testing. The upgraded FLEXIFIT program demonstrated the superiority of the non-parametric curve shape estimation in giving

significantly greater accuracy to radioimmunoassay; assays analyzed by FLEXIFIT will no longer be constrained by the need to find linear or logistic dose-response curves. Existing radioimmunoassay dose interpolation software was modified to incorporate the FLEXIFIT approach; the software is now available for use on microcomputer. In an attempt to improve statistical analysis of pulsatile hormone release, new methods based on the principles of deconvolution have been developed to detect peaks of episodic hormone secretion, to derive the instantaneous rate of hormone secretion, and to estimate the kinetics of hormone clearance. The limitations of previous methods have been examined. New methods have been developed to evaluate the concordance of episodic events in two hormonal time series. This will facilitate the investigation of co-secretion of hormones (e.g., LH, FSH, LH  $\alpha$ -subunit, and prolactin) and of the relationship between input and output of a gland (e.g., LH, testosterone). The theoretical basis for analysis of hormone receptor systems has been expanded. Also based on deconvolution, a new method has been developed to derive a two-dimensional affinity spectrum for one labeled ligand and two unlabeled ligands reacting with any number of classes of binding sites.

Theoretical developments were made to provide optimal design of experiments for systems involving one or two classes of binding sites. When more than one binding site is present, a dose response surface for two ligands can give considerably more information than a conventional self- and cross-displacement experiment. A new computer program "DESIGN" calculates the optimal combinations of ligand concentrations on this surface. This program can handle multiple ligands, multiple classes of sites and dose response surfaces and competitive ligands. The program and experimental strategy have been applied successfully to the characterization of glucocorticoid receptors in the hypothalamus (in collaboration with DEB). Together with LIGAND, a program developed earlier by this group, DESIGN was tested in laboratory studies by Rodbard and Munson and their coworkers by applying it to the characterization of receptors for phencyclidine (PCP) and sigma ligands (SKF10,047) in membranes prepared from rat and guinea pig brain. They found that the PCP receptor is present in both high and low affinity forms. The high affinity form is stabilized by L-glutamate and thus appears to be a site in the ion channel allosterically modulated by excitatory amino acid receptors of the NMDA type. Evidence was also obtained that homocysteic acid may be a naturally occurring agonist at this site. Its activity is virtually identical to that of L-glutamate, shows stereospecificity, and is appropriately modified by allosteric regulators and antagonists at the NMDA binding site.

In a new project directed by Juan Calvo, cell-cell communication between normal mammary epithelium and connective tissue is being studied, using 3T3 fatty fibroblasts and 3T3-L1 preadipocytes *in vitro*. Adipocytes and fibroblasts were found to stimulate the rate of proliferation and alter the morphology of mammary epithelium. Further, the mammary epithelium produces a factor, present in conditioned medium, which results in inhibition of the transformation of 3T3-L1 cells to adipocytes. This can be measured morphologically, by Oil-Red-O staining, or by measurement of triglyceride accumulation. Current studies are underway to characterize this factor (or factors) biochemically.

The interests of members of the Section on Metabolism and Mass Spectrometry, led by Alfred Yergey, are concentrated in two main areas—the application of thermal ionization mass spectrometry to studies of calcium metabolism in neonates, infants, normal children, and during pregnancy and lactation, and the development and application of thermospray liquid chromatography-mass spectroscopy (LC/MS) to the study of metabolism of glucose, Vitamin D, cortisol, testosterone, and small peptides. The Section is one of the few laboratories in the world conducting research on the clinical applications of stable isotopes.

One of the main objectives of the work is to elucidate metabolic kinetics for calcium in populations that are inaccessible to study with radiotracers, particularly children and women of childbearing age. To date, this objective has been met by studies of the kinetics of calcium distribution both in normal children and in children with disease related to abnormal calcium metabolism, as well as in normal lactating and nonlactating women. The mass of calcium (Ca) in the rapidly exchanging internal pool (MPCa) has been determined from the

zero time intercept of a sum of exponentials, fit to the data obtained from the time dependent dilution of an intravenously administered tracer. This pool size has been shown to differ substantially from values expected if the pool were assumed to be a fixed fraction of body mass. The differences from the expected values in normal children (aged 2 wks - 14 yrs) correlate significantly ( $r=0.95$ ,  $p<0.01$ ,  $n=10$ ) with increase in bone mineral content (mg/cm/wk). This suggests that this pool is an indicator of physiologically active bone mass or bone formation and that it is a potential predictor of skeletal growth in children and bone loss in older subjects.

A modification of this methodology permits the measurement of true fractional absorption of calcium. Studies of fractional absorption of dietary calcium in normal adult women have shown excellent agreement between radioactive and stable isotope tracer methods. When adjusted for dietary intake, these results show a total absence of age dependence for calcium absorption. In addition, age matched women with osteoporosis absorb calcium at the same level as normal women, but appear to have an increased rate of urinary calcium excretion. Premature infants are at risk for low bone mineralization, because 80% of the bone calcium is deposited in the third trimester. Therefore, other methodological modifications have been made to assess the efficiency of calcium absorption in premature infants under various feeding protocols. The method is sufficiently benign to be used in infants and allows the determination of endogenous fecal excretion of calcium, along with urinary and total fecal calcium excretion, which give an estimate of calcium retention. The results of these investigations suggest that more calcium is being retained by these infants than had previously been believed. This may affect feeding protocols for these infants.

The Section has been at the forefront of developments in Thermospray LC/MS and its biological applications. New technology has been developed to measure biological materials that require mass spectrometric analysis, but which have been difficult or impossible to analyze by other methods because of the thermal lability, involatility or charge state of the molecule. Thermospray LC/MS allows the measurement of true cortisol production rates (FPR), rather than deriving them from plasma cortisol concentrations. Surprisingly, normal adults were found to have an average value of  $9.6 \pm 2.6$  mg/24 hrs, about 50% lower than the previously generally accepted value. The anticipated circadian variation was observed, and the narrow range of values is consistent with the remarkably low variation observed in mean daily plasma cortisol concentration ( $[F] = 6 \pm 0.9$   $\mu$ g/dl) determined by the isotope dilution methodology developed by this Section. FPR and plasma cortisol values are highly correlated in normal volunteers, indicating that hormone secretion, rather than its metabolism, is responsible for the changes in plasma level.

The methodology has been validated in six adrenalectomized patients by infusing a known amount of cortisol at known ratio of labeled to unlabeled material over the course of a day. Daily FPR differed by less than 6% from expected. The tracer molecule contains deuterium, which would be expected to cause some perturbations of the molecular structure, but no isotope effect was observed *in vivo*, i.e., the ratio of labeled to unlabeled material observed in plasma did not differ from that in the infusate. Furthermore, based on *in vitro* studies, no isotope effect is seen in solvent extraction. In 19 patients with surgically proven Cushing's Syndrome, the method showed an increased FPR, accompanied by loss of diurnal rhythm of cortisol excretion. Both the 24 hr and 8 pm-midnight FPR allow separation of normal subjects from those with Cushing's Syndrome. In addition, urinary 17-hydroxycorticosteroid excretion was seen to correlate better with FPR than the customary urinary free cortisol measurement, suggesting that the former is a more sensitive and specific diagnostic test. FPR was also measured in 33 healthy children aged 8-18. Overall, FPR was found to be  $9.5 \pm 2.5$  mg/24 hr ( $6.8$  mg/m<sup>2</sup>/24 hrs.). This is approximately half the currently accepted value. This may explain why many children with adrenal insufficiency grow poorly when treated with glucocorticoid replacement doses. The replacement dose should be re-evaluated carefully. There are no gender differences, nor does FPR vary with stage of puberty.

The Section on Macromolecular Analysis, under the direction of Andreas Chrambach, is engaged in studies of gel electrophoresis of proteins, nucleic acids, and protein-nucleic acid

complexes. This year, the Section turned its attention to the development of gel electrophoretic methods for obtaining the size, conformation and surface net charge of DNA and DNA-protein interaction products. The Section has made four major contributions to this field: i) extension of the DNA size range amenable to polyacrylamide gel electrophoresis significantly beyond previous limits; ii) introduction of discontinuous buffer systems into DNA electrophoresis; iii) application of Ferguson plot (log mobility vs gel concentration) analysis to the determination of the conformational consequences of protein binding to DNA; iv) analysis by Ferguson plot of the conformational consequences of variations in electrophoretic conditions on the conformation of DNA.

To date, DNA up to 1 kbp has been routinely analyzed by conventional, relatively restrictive, polyacrylamide gels, while the less restrictive agarose can be used up to 25 kbp. Using highly crosslinked polyacrylamide (a high degree of crosslinking increases pore size), Chrambach and his colleagues were able to extend the DNA size range applicable to PAGE to 10-20 kbp. Compared with agarose, polyacrylamide has the advantage of sharper zones and higher resolution, relative absence of zone perturbations and compatibility with discontinuous buffer systems. Moreover, polyacrylamide gel fibers are not oriented by electrophoresis as are agarose fibers, and polyacrylamide has the advantage of not exhibiting electroendosmosis, which tends to broaden bands. Discontinuous buffer systems were applied to DNA electrophoresis for the first time, and have the advantage of providing sharp starting zones, which improve resolution, and of ensuring electrophoresis of every component of the mixture, of providing a moving boundary front of constant sharpness as a reference for calculations of mobility, and of freeing mobility determinations of their dependence on time and field strength.

These improvements have facilitated the application of Ferguson plot analysis to DNA. Ferguson plot analysis is important because it allows one to determine the degree to which molecular size and/or net charge contribute to migration rates, independently of standards. It thus provides an experimental tool to test the widely held assumption that the migration rate of DNA is solely governed by size. Conformational alterations, bound proteins and counterion compositions, which vitiate that assumption, can be detected by Ferguson plot analysis. In collaboration with Drs. Zwieb and Adhya of NCI, subtle alterations in DNA conformation and of DNA/protein interactions were detected by determining the size and net charge of two variants of the Gal operator 18 bp sequence bound to the Gal repressor, which were centrally embedded in a 155 bp DNA fragment of pBR322. Ferguson plot analysis permitted conclusions to be drawn about the effects of these variations on the molecular surface area of these complexes. It is also possible to move the operator sequence to the periphery of the 155 bp fragment, which results in increased electrophoretic mobility, due not to increased size but to increased charge density. All protein binding was found to result in a diminished surface net charge. These findings were made possible by another new method devised in the Section, *viz* the measurement of mobility in copolymers of polyacrylamide with agarose. This technique, by providing mobility data in a non- or minimally sieving medium overcomes the ambiguity and error involved in the determination of free mobility (surface net charge) by extrapolation of linear Ferguson plots to zero gel concentration.

Other work is in progress to determine by Ferguson plot analysis the conditions of electrophoresis (gel concentration, ionic strength, temperature and field strength) under which the random coil conformation of DNA is preserved (reptating DNA cannot be separated by gel electrophoresis without pulse power). A surprising finding was that the DNA-ethidium bromide complex shrinks as the concentration of ethidium bromide in the complex is increased. Apparatus design improvements were aimed at a gel electrophoresis apparatus optimized for "Ferguson plot analysis", i.e., simultaneous electrophoretic separations at several gel concentrations, at the nanogram load level, avoiding gel dehydration ("submarine") and adaptable to discontinuous buffer systems. Work was continued on the elaboration of an operationally simplified technique of Immobiline electrofocusing. Thus, the Peltier-cooled apparatus used for that purpose was adapted for focusing on immobilized pH gradients under a layer of silicone oil. This prevents water exudation and dehydration

problems, and allows for the long duration of focusing required to obtain steady-state gel patterns. Further, conditions for diffusion blotting of Immobiline gels were found that circumvent the electroendosmotic problems of electrotransfer of zones from such gels. The method has been applied successfully to the determination of the carbohydrate composition of an 18-component acid phosphatase.

A problem of electrophoresis hitherto considered intractable is the physical characterization of material that exhibits a "smear" pattern, a characteristic of polydisperse populations of molecules, rather than a distinct band pattern. The Section has developed a computer method for evaluating polydisperse mixtures of subcellular-sized particles with regard to size and surface net charge. A program was developed for the Macintosh II computer, which is capable of determining the relative abundance of each component species in such polydisperse mixtures, together with its size and net charge, using the input of a two-dimensional, densitometrically analyzed, gel pattern. In a pilot study, conducted in collaboration with the LDMI, this approach is being applied to the analysis of the relationship between immunogenicity and electrophoretically determined physical properties of vaccines made by conjugation of bacterial polysaccharides with proteins.

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## HUMAN GENETICS BRANCH

William A. Gahl, M.D., Ph.D., Chief



Left to right: J. Chou, E. Butler, W. Gahl, I. Owens, J. Marini

The Human Genetics Branch conducts both clinical and basic research directed toward elucidating the causes and finding optimal treatments for human genetic diseases. Clinical research projects focus largely upon the inborn errors of metabolism and other heritable disorders. Particular emphasis is placed on determining the natural history and diagnosis of, and on developing innovative therapy for, children suffering from rare diseases. Bench work employs *in vitro* systems to study cell biology, with special emphasis on models of human development and genetic disease. The Human Genetics Branch has firmly founded itself in basic research, concentrating on the application of current research techniques to the clinical conditions under investigation. Conversely, the human mutations under study provide a basis for the cell biological and developmental aspects of the Branch's research. One Section and one Unit in the Branch have dual responsibilities for patient care as well as basic research; these are the Section on Human Biochemical Genetics and the Unit on Connective Tissue Disorders.

The Section on Human Biochemical Genetics, under the direction of **William Gahl**, studies rare inborn errors of metabolism both in the patients and at the bench. A great deal of effort is invested in the care and investigation of children and young adults with nephropathic cystinosis. This autosomal recessively inherited disorder results from defective transport of the disulfide amino acid cystine out of lysosomes and into the cytoplasm of cells. The accumulation and crystallization of cystine destroys several tissues, and classically causes death from renal failure by ten years of age. Renal transplantation resolves the kidney problem, but continued cystine accumulation progressively destroys other organs. Therapy is directed at reducing the cellular cystine content. This is accomplished by administering oral cysteamine, an aminothioli whose cystine-depleting effects have proven efficacy in improving growth and maintaining renal glomerular function. The Section cares for one third of the 200 patients in the United States and serves as a national referral and international resource center regarding this disease. Pre-transplant patients receive oral cysteamine (or the

more palatable phosphocysteamine) in standard-dose or in high-dose, as part of a national clinical trial to determine the optimal dosage regimen of this drug. The Section is the only group in the country to offer cysteamine to post-transplant patients as well.

Infants with cystinosis have renal tubular Fanconi syndrome, in which small molecules fail to be reabsorbed by the kidney and are excreted in the urine. One of the issues in cystinosis treatment is whether early administration of oral cysteamine can prevent the Fanconi syndrome. One patient has been treated with cysteamine from 14 days of age; this boy is now 24 months old with a very mild Fanconi syndrome and remarkably good growth compared to that of his older affected brother, in whom treatment was begun later in life. The treatment of Fanconi syndrome itself has been improved by providing oral L-carnitine supplementation (at 100-200 mg/kg/day) to affected children, whose kidneys fail to reabsorb L-carnitine and, therefore, have low plasma and muscle levels of this compound. L-carnitine is essential for the transport of fatty acids into the mitochondria for subsequent oxidation and energy production. The Section is conducting a long-term trial of L-carnitine supplementation to determine whether muscle carnitine can be repleted and muscle strength and integrity improved by five years of replacement therapy.

Patients with cystinosis develop cystine crystals in the cornea by approximately one year of age. These crystals are refractory to oral cysteamine therapy, but 0.1% (10 mM) cysteamine eyedrops, given hourly while awake, can dissolve these crystals in very young children. Older patients, with more crystals, are poorly responsive. Therefore, in conjunction with Dr. Kaiser-Kupfer of the National Eye Institute, and after demonstrating the range of toxicity of topical cysteamine in rabbit eyes, the concentration of cysteamine eyedrops administered to cystinosis patients was increased to 0.5%. The Section now has seven children, age 2-5 years, who have objective evidence of clearing of corneal crystals. This may prove of long-term benefit to these patients, because the crystals eventually become so packed that they produce a hazy cornea and recurrent corneal erosions. Cysteamine eyedrop therapy is now being extended to include children who have even more densely-packed corneas.

Members of the Section on Human Biochemical Genetics have also recognized the occurrence of serious non-renal complications in cystinosis. While caring for 30 post-renal transplant patients, they discovered a cystinosis myopathy that involves muscle weakness and atrophy and the risk of aspiration. In conjunction with Dr. Barbara Sonies of the Rehabilitation Medicine Department, it was discovered that the majority of patients over ten years of age have swallowing abnormalities. Several patients with cerebral atrophy on CT scan and three patients who have paraventricular and basal ganglia calcifications have been reported. In the past year, the first case of pregnancy in a cystinosis woman was documented; she delivered an entirely normal male infant despite having cystine crystals in the maternal, but not the fetal, portion of her placenta. Virtually all cystinosis patients have delayed puberty, and males have primary hypogonadism and aspermia. This work was done in conjunction with Drs. Chik and Merriam of the Developmental Endocrinology Branch of NICHD. All these efforts at defining the natural history of cystinosis are the first steps to improve the care of patients with this multisystemic disease.

The Section has taken a recent interest in the study of the neurological, renal and ophthalmic complications of Lowe (oculocerebrorenal) syndrome, in an attempt to characterize its full phenotypic variation. Within the past year, Dr. Lawrence Charnas has studied 25 males with this X-linked disorder and reported variable degrees of central demyelination, detected by CT and MRI scans, in these patients. He has also found three patients who had dermoid tumors of unknown etiology. All of the patients displayed marked proteinuria, with a characteristic pattern distinguished by prominent bands in the gamma region on urine protein electrophoresis. The particular type of Fanconi syndrome in Lowe syndrome patients is primarily proteinuria, with a mild to moderate loss of small molecules such as phosphate, amino acids, and carnitine. The recruitment of Lowe syndrome patients to the NICHD permits the definition of the natural history, and provides material for the investigation of the basic defect in this disease. This is being approached through measurement of nucleotide pyrophosphatase, an enzyme reported elevated in Lowe syndrome fibroblasts. The patient

population also provides DNA for future molecular biology investigations into the Lowe syndrome gene, which is located on Xq 24-26. Family studies are also being performed, since lenticular opacities have been identified in several obligate heterozygotes for the disease.

Members of the Section have reported the results of treating 5-pyridoxine-nonresponsive homocystinuria patients with oral betaine at 3 grams b.i.d. A two year, double-blind, placebo-controlled crossover study showed that betaine, which aids in remethylating homocysteine to methionine, did not improve bone density in the homocystinurics. In another project, Dr. Henk Blom, a Visiting Fellow, showed that a product of methionine transamination, dimethylsulfide, was elevated in patients with methionine adenosyltransferase deficiency, as well as in a boy with hypermethioninemia due to cystathionine  $\beta$ -synthetase deficiency. This establishes the existence of the transamination pathway in methionine metabolism.

Members of the Section have diagnosed a 21 month-old boy with severe liver disease and liver copper storage as having Indian Childhood Cirrhosis (ICC). This is the second case of ICC reported in the United States. The findings of electron-dense aggregates in cultured fibroblasts and consanguinity in the family indicate the genetic nature of this defect, which was previously considered to be environmental and confined to India. Analysis of plasma amino acids in fetal blood samples, obtained by cordocentesis by Isa Bernardini, is aimed at establishing normal values for fetuses at different gestational ages. This work is being carried out in collaboration with Dr. Nicolaides of King's College in London, and will prove valuable for future diagnosis of aminoacidopathies by cordocentesis. Bernardini also found that six amino acids, i.e., lysine, valine, threonine, methionine, tyrosine, and phenylalanine, are concentrated by the human fetus, while nonessential amino acids are not. Some of the blood samples were from intrauterine growth retarded fetuses, and five of these samples failed to concentrate even the essential amino acids mentioned above. It was also determined that carnitine is not concentrated by the normal fetal circulation.

The Section on Human Biochemical Genetics has not limited itself to clinical work. Members of the Section study sialic acid metabolism from a standpoint of various human mutations. One of these is infantile free sialic acid storage disease (ISSD), a rare lysosomal disorder in which free sialic acid accumulates within lysosomes. ISSD fibroblasts were found to store within their lysosomes another charged sugar besides sialic acid, namely, glucuronic acid. This suggests that both glucuronic acid and sialic acid are transported by the same carrier, known to be defective in ISSD. Members of the Section also had access to two cell strains in which sialic acid accumulated in the cytosol rather than the lysosomes. These patients have sialuria, and in collaboration with Drs. Gil Ashwell, Peretz Weiss, and Frank Tietze of the NIDDK, it was determined that this disorder is caused by a failure of CMP-sialic acid feedback to inhibit the initial, regulatory enzyme in sialic acid synthesis, UDP-N-acetylglucosamine 2-epimerase. This is an extremely rare inborn error, which is due to defective allosteric inhibition with preservation of the catalytic activity of the enzyme. The Section has also studied patients with sialic acid disorders to determine how the human kidney handles sialic acid. It was proved that this charged sugar is filtered but not reabsorbed.

The Section continues its investigation into lysosomal transport of small molecules. One specific pursuit is the study of a transport system for tyrosine and neutral amino acids, system h, which was characterized in rat FRTL-5 thyroid cells. Tyrosine, phenylalanine, and leucine transport are each stimulated 3 to 7-fold by thyroid-stimulating hormone (TSH) in the system. This makes system h the first integral lysosomal membrane carrier shown to be hormonally responsive. System h also carries monoiodotyrosine (MIT) across the lysosomal membrane. MIT is produced by lysosomal hydrolysis of thyroglobulin. The iodine is salvaged in the cytosol for reutilization, and this requires transport of MIT across the lysosomal membrane. The MIT carrier is also stimulated by TSH, and appears to recognize diiodotyrosine (DIT). This allows the use of  $^{125}\text{I}$ -DIT as a natural photoaffinity label, which binds covalently to molecules in its vicinity upon exposure to ultraviolet light. In collaboration with Dr. Len Kohn of the NIDDK,  $^{125}\text{I}$ -DIT was found to bind to a 70 Kd

protein, whose N-terminus was sequenced. A 53-mer candidate oligonucleotide was synthesized and served as a probe against an FRTL-5 library. Positive colonies are now being screened in a procedure that may help isolate the gene for the lysosomal MIT/DIT carrier. A primary goal of the Section is to isolate a functional integral lysosomal membrane protein, e.g., a carrier protein.

In a separate project, **Jean Butler** is studying a mutant mouse with a defect in cholesterol esterification and with lysosomal accumulation of cholesterol. The phenotype of this mouse resembles Niemann-Pick C disease, as studied in human fibroblasts, which also store large amounts of cholesterol in their lysosomes and Golgi complexes. Dr. Butler and her collaborators have shown that HMG CoA reductase, a rate limiting enzyme for cholesterol anabolism, and receptor-mediated LDL uptake are normal in Niemann-Pick C cells. Investigation of these cells and the mutant mouse model may help elucidate the pathway of cholesterol movement within cells and identify the defect in Niemann-Pick C disease.

The second organization within the Human Genetics Branch with both patient care and basic research responsibilities is Joan Marini's Unit on Connective Tissue Disorders. Dr. Marini studies the molecular basis of heritable disorders of connective tissue and their treatment. The Unit focuses its efforts on osteogenesis imperfecta (OI), a generalized connective tissue disorder, whose hallmark is a tendency to fracture bones easily. OI has been associated with defects in the structure and function of type I collagen, a major protein of bone and skin. Dr. Marini cares for a total of 50 children with OI under two clinical protocols. One protocol enrolls 25 children and involves the evaluation of endocrine parameters of growth failure in OI. This study has produced the only known description of the function of the growth hormone axis in a primary bone disorder; the data are now being compared with those generated by the Developmental Endocrinology Branch of NICHD regarding normal children. In another protocol, in collaboration with the Rehabilitation Medicine Service of the Clinical Center, the Unit continues to study the effect of lower limb bracing on ambulation in children with moderately severe OI.

In the area of basic research, the Unit has developed a system for detecting point mutations in type I collagen mRNA by RNase A digestion of mismatches in RNA/RNA hybrids between anti-sense riboprobes and the mRNA of patients. This anti-sense riboprobe system now covers the entire mRNA for the two genes coding for type I collagen. The Unit has also developed a complementary methodology for the rapid isolation and sequencing of point mutations, which have been detected by the RNA/RNA hybrid system. This approach utilizes amplification of the target region by the polymerase chain reaction (PCR). The patient's poly (A<sup>+</sup>)-RNA is used for the first strand synthesis of cDNA. This DNA/RNA hybrid is used directly as a substrate for PCR. After amplification of the mismatch-containing region, the amplified fragments are subcloned into appropriate vectors and sequenced.

The riboprobe system has been applied to the mRNA of patients with osteogenesis imperfecta, and mismatches have been detected in patients with Types II, III and IV OI. The point mutations have been isolated and sequenced in a case of moderate type IV OI and a case of lethal type II OI. In each case, the mRNA data are in full agreement with the collagen protein data on the patient. In the Type IV case, a single nucleotide change, G→A, results in the substitution of a serine for a glycine in amino acid residue 832 of one allele of the  $\alpha 1(I)$  chain. In the Type II case, a G→A change results in the substitution of an aspartic acid for a glycine at amino acid residue 805 of one allele of the  $\alpha 2(I)$  chain. By contrast, previous models had suggested that substitutions for glycine in the  $\alpha 1(I)$  chain would be lethal, while those in the  $\alpha 2(I)$  chain would cause a non-lethal phenotype. This data extends our understanding of the molecular basis of OI and requires alteration of existing models.

The Unit continues to conduct molecular analyses of patients with connective tissue disorders. Additional mutations in type I collagen of OI patients will be isolated and sequenced, including an unusual case of dominant Type III OI. The Unit also plans to initiate an

RNA/RNA hybrid system for Type III collagen, and to use it to study the molecular defects in patients with Ehlers-Danlos syndrome.

James Sidbury, an Adjunct Scientist in the Human Genetics Branch, continues his studies of glucose metabolism in patients with glycogen storage disease. Adult patients with type I glycogen storage disease due to glucose-6-phosphatase deficiency have been shown to produce glucose at a rate approximating that of normal fasting adults. Dr. Sidbury is also investigating which regimen of raw starch will maintain the best and longest blood glucose levels in patients with glycogen storage disease.

Janice Chou leads the Section on Cellular Differentiation, which attempts to understand the regulation of gene expression during normal and abnormal differentiation. The major areas of emphasis are: expression of the  $\alpha$ -fetoprotein (AFP) gene in liver; hormonal regulation of liver genes in differentiated hepatocyte lines; establishing mouse liver cells carrying genetic mutations; cloning and expression of the human pregnancy-specific  $\beta_1$ -glycoprotein (PS $\beta$ G) gene; and cloning and expression of the human alkaline phosphatase (AP) gene.

Over the past several years, the Section has studied expression of the AFP gene in fetal and adult rat livers, and in temperature-sensitive hepatocyte lines established by this group. They found that adult rat livers express three AFP mRNAs of 2.2 kb (minor), 1.7 kb, and 1.5 kb, whereas fetal livers express a 2.2 kb AFP mRNA. A similar 1.7 kb AFP mRNA is also expressed by the SV40-transformed fetal and adult hepatocyte lines, and several hepatoma cell lines. The structure of the 1.7 kb transcript was determined by isolating and characterizing cDNA clones, encoding the 1.7 kb variant AFP, from cDNA libraries of adult rat liver and a RLA209-15 fetal hepatocyte line. Sequence analysis indicated that the 2.2 kb and 1.7 kb AFP mRNAs share 1.1 kb of 3' sequences, but that they differ in sequences at the 5' regions. The 5' exon (V) of the 1.7 kb mRNA, which is absent from the 2.2 kb AFP mRNA, is located in the seventh intron of the rat AFP gene. Since there is only one AFP gene per haploid genome in rat, these results suggests that the two transcripts evolved by the alternative use of promoters.

The group also demonstrated that alkaline phosphatase (AP) in human choriocarcinoma cells (malignant trophoblasts) differs from placental AP by its greater sensitivity to EDTA and L-leucine inhibition, and by its greater electrophoretic mobility on polyacrylamide gel. It was found that choriocarcinoma cells express a 2.6 kb AP mRNA, unlike normal human placenta, which expresses a 2.8 kb AP mRNA. Administration of sodium butyrate to choriocarcinoma cells greatly increases the steady-state levels of the 2.6 kb choriocarcinoma AP mRNA, resulting in increased enzyme activity and biosynthesis. S1 nuclease analysis, using probes derived from a placental AP cDNA, and ribonuclease protection assays, employing probes derived from the germ-cell AP gene, demonstrated that choriocarcinoma cells express the germ-cell AP gene. The germ-cell AP gene encodes the placental AP-like isozyme, which is primarily expressed in the thymus, testis, and germ-cell tumors. The structures of the internal exons (II through X) of the germ-cell AP gene were determined previously, based on their similarity to the placental AP gene. However, the boundaries of exons I and XI (3' exon) of the germ-cell AP gene were not defined, because of sequence divergence between the two genes in the 5' and 3' regions. Using the ribonuclease protection and primer extension assays, Chou and coworkers demonstrated that exon I of this gene is 119 bp in length and that germ-cell AP mRNA contains one major transcription initiation site. In addition, they isolated and characterized germ-cell AP cDNA clones from a butyrate-treated choriocarcinoma cDNA library, and showed that the germ-cell AP mRNA is 2,487 bp long and that exon XI of this gene is 1135 bp long.

Chou and coworkers have isolated and characterized four distinct cDNA clones encoding human pregnancy-specific  $\beta_1$ -glycoprotein (PS $\beta$ G). Two types of PS $\beta$ Gs are encoded by these cDNAs: those that contain two repeated protein domains (1a and 2a) and those that contain only one protein domain. All the encoded PS $\beta$ Gs so far analyzed differ in sequence at the carboxyl terminus, indicating that PS $\beta$ G is a very polymorphic protein. Chou's group confirmed this by identifying multiple PS $\beta$ G genes in the human genome. They also showed

that PS $\beta$ G shows strong sequence homology to human carcinoembryonic antigen (CEA), which, along with PS $\beta$ G, belongs to the immunoglobulin gene superfamily. CEA has been shown to be an intercellular adhesion molecule. Chou and coworkers are currently designing experiments to investigate the possible roles of PS $\beta$ G as an intercellular adhesion molecule.

The Section has also demonstrated that human placental fibroblasts produce a PS $\beta$ G immunologically indistinguishable from placental PS $\beta$ G. They subsequently confirmed this finding by the immunocytochemical localization of PS $\beta$ G in these fibroblasts. Chou and coworkers found that the major PS $\beta$ G species synthesized by placental fibroblasts is a 62K glycoprotein, whereas the PS $\beta$ G species found in human placenta consists of one major 72K glycoprotein and two minor 64K and 54K glycoproteins. Thus, the major PS $\beta$ G species found in fibroblasts and human placenta differ. Synthesis of the 62K PS $\beta$ G species by fibroblasts is slightly stimulated by sodium butyrate. However, butyrate-treated fibroblasts produce greatly increased amounts of 48K and 72K PS $\beta$ Gs. The similarity in PS $\beta$ Gs produced by butyrate-treated fibroblasts and placenta was confirmed by cell-free synthesis of PS $\beta$ G. Chou's group previously isolated three PS $\beta$ G cDNA clones (PSG16, PSG93, and PSG95), which share similar sequences in the 5' region (designated PSG-5'), but differ in sequences in the 3' regions. PSG16 and PSG93 differ only in that PSG93 contains an additional 86-bp sequence at nucleotide 1308 of PSG16. PSG95 contains a 3' untranslated region which differs from that of PSG16/PSG93. Probes derived from these cDNAs hybridize with three PS $\beta$ G mRNAs of 2.3 kb, 2.2 kb, and 1.7 kb in placental fibroblasts, and sodium butyrate increases the steady-state levels of all three mRNAs. In butyrate-treated fibroblasts, the PS $\beta$ G mRNAs that contain the PSG-5' or PSG93-specific sequence are increased to approximately 20% of the levels in human placenta. However, unlike human term placenta, which expresses predominantly PS $\beta$ G mRNAs with 3'-sequences similar to PSG16/PSG93, the butyrate-treated fibroblasts express roughly equal levels of PS $\beta$ G mRNAs with 3' ends of PSG16/PSG93 and PSG95. Control fibroblasts mainly express a placental PS $\beta$ G mRNA that shares part of PSG93-specific sequences. This species is only slightly enhanced by butyrate, and it may therefore be the mRNA that encodes the 62K fibroblast PS $\beta$ G. The finding that a variant PS $\beta$ G species is produced in placental fibroblasts suggests that the authentic placental PS $\beta$ G species may have different functions.

Chou and coworkers have also examined the expression of albumin, tyrosine aminotransferase (TAT), and phosphoenolpyruvate carboxykinase (PEPCK) genes in an adult rat hepatocyte cell line, RALA255-10G, which is temperature sensitive and dependent upon glucocorticoid hormone for differentiation. They found that expression of albumin and TAT genes requires the continued presence of glucocorticoid hormone, and that this expression is enhanced by cAMP, although cAMP alone does not enhance albumin or TAT synthesis. Expression of the PEPCK gene is stimulated by cAMP and glucocorticoid hormone. Administration of retinoic acid inhibits the glucocorticoid-mediated induction of both albumin and TAT genes, but stimulates the cAMP/glucocorticoid-mediated induction of the PEPCK gene. Transcriptional regulation is the primary site of action. This cell line, established by Chou's group, provides an excellent model to examine mechanisms regulating liver differentiation by glucocorticoids, cAMP, and retinoic acid.

Chou and coworkers have recently established mouse liver cell lines carrying deletions near the albino locus on chromosome 7. These mouse mutants express low levels of several liver-specific genes, including PEPCK and TAT. It was proposed that a trans-acting control function, localized on chromosome 7 near the albino locus, is required for expression of many liver genes. These cell lines were established by transforming primary liver cells with temperature-sensitive A (tsA) mutants of SV40; these are temperature sensitive in the gene required for the maintenance of transformation. The mouse liver cell lines, which are ts for growth and differentiation, express a variety of mouse liver genes including albumin, transferrin, TAT, and PEPCK. In contrast to *in vivo* studies, which showed that the albino deletion mutant mouse expresses low levels of TAT and PEPCK genes insensitive to glucocorticoid and cAMP induction, mouse liver cell lines carrying the albino deletion express both TAT and PEPCK genes; the expression is greatly increased by glucocorticoid hormone and cAMP. These findings indicate that the *trans*-acting factor, which is involved

in control of liver gene expression in the albino deletion mouse mutants, may not be located in chromosome 7. Alternatively, control of liver gene expression may involve the interaction of multiple factors, including the *trans*-acting factor encoded by chromosome 7, which cannot be studied in an isolated cell line.

The Section on Developmental Genetics, under Anil Mukherjee, conducts both basic and clinically related research into the biochemistry, molecular biology and genetic regulation of the enzyme phospholipase A<sub>2</sub> (PLA<sub>2</sub>) and its inhibitory proteins with antiinflammatory and/or immunomodulatory properties. Inflammatory diseases are a major and important cause of human morbidity and mortality, and even some presumably non-inflammatory diseases have an inflammatory component. Although excessive inflammation contributes to many disease processes, absence of this phenomenon may lead to a compromised host. Thus, inflammation is essential, ongoing, and a precisely regulated process in a healthy individual. One of the pathways for the initiation of an inflammatory process is the activation of a lipolytic enzyme, phospholipase A<sub>2</sub> (PLA<sub>2</sub>). This enzyme may also be involved in many other physiological functions, including cellular signal transduction and immunological activation. PLA<sub>2</sub> is a key enzyme in the production of arachidonic acid from cell membrane phospholipids, and plays a regulatory role in the production of eicosanoids, e.g., prostaglandins, leukotrienes and thromboxanes. Some of these eicosanoids are known mediators of inflammation.

For the past 11 years this group has been engaged in investigations that led to the discovery of a steroid-dependent, small molecular weight, secretory protein called uteroglobin (UG), which is an inhibitor of PLA<sub>2</sub>. Last year the group identified two nonapeptides, MQMKKVLDS from UG and HDMNKVLDL from lipocortin-1, which are potent PLA<sub>2</sub> inhibitors and antiinflammatory agents. These oligopeptides appear to represent the active site, or a portion of the active site, responsible for the PLA<sub>2</sub> inhibitory activity of the proteins from which they are derived. To demonstrate this more conclusively, the group will perform site-directed mutagenesis involving large scale expression of this protein in *E. coli*. However, this poses a unique problem, since the protein is a dimer with two interchain disulfide bridges connecting the monomers; to date, it has not been possible to express a eukaryotic protein containing two disulfide bridges in a bacterial host. To overcome this problem, the Section successfully designed an expression vector, pLE103-1, in which the expression of recombinant UG is controlled by a bacteriophage T7 late promoter. With pLE103-1, recombinant UG production reached approximately 9-11% of total bacterial soluble proteins. This recombinant protein has been purified to homogeneity. Its N-terminal amino acid sequence confirmed that the protein is identical to natural rabbit UG, and it forms disulfide bridges at the appropriate amino acid residues, i.e., Cys 3' to 69 and 3 to 69' residues. The recombinant UG was also found to be as active a PLA<sub>2</sub> inhibitor and antithrombotic agent as its natural counterpart. The successful development of this expression system is now being used for site-directed mutagenesis studies. Because of its potential biomedical and commercial possibilities, a patent application has been filed for this vector.

The Section has also used a monospecific polyclonal antibody to rabbit UG to screen human lung and prostatic cDNA libraries ( $\lambda$ gt11). This yielded several positive plaques. Plasmids from one of these plaques were partially characterized and found to contain a 500 bp human cDNA, which is now being sequenced. If this fragment consists of a unique sequence, the group will be able to clone a human UG cDNA. In collaboration with Dr. David Bullock of Lincoln College, Canterbury, New Zealand, the group has generated transgenic mice by pronuclear microinjection of cloned genomic UG DNA including a 2.3 kb 5'-flanking sequence. UG-mRNA can be detected in the lung and the uterus but not in liver, kidney, brain or spleen of transgenic mice. The level of expression of UG-mRNA in transgenic mice lungs exceeds that in the rabbit while uterine expression is weaker. Strong immunofluorescence is present in the bronchial and pulmonary alveolar epithelium, but is not detectable in the endometrium. Since UG has profound antiinflammatory properties, these mice may provide a valuable model for studies of pulmonary inflammation.

The antiinflammatory peptides described last year (e.g., MQMKKVLDS and HDMNKVLDL) have a shared essential sequence KVLD (P4). This tetrapeptide has no anti-PLA<sub>2</sub> or antiinflammatory activity. However, it is a potent inhibitor of platelet aggregation induced by ADP (IC<sub>50</sub> = 3-6 x 10<sup>-4</sup>M) and thrombin (IC<sub>50</sub> = 6.8-7.4 X 10<sup>-4</sup>M). Although MQMKKVLDS (P1) and P4 inhibit thrombin esterolytic activity, aggregation induced by thrombin is inhibited less effectively than aggregation induced by ADP. Serotonin secretion by platelets is not affected, despite inhibition of aggregation. This indicates that the most significant mechanism of inhibition involves a step subsequent to full platelet activation. Platelet binding of <sup>125</sup>I-fibrinogen was found to be inhibited by P4 (K<sub>i</sub> = 9 x 10<sup>-4</sup>M). When P1 and P4 peptides are used together there is an additive effect on inhibition of aggregation. This suggests that there are two mechanisms involved, i.e., that P1 works by inhibiting PLA<sub>2</sub>, a necessary step in the activation of platelets, and that P4 works by blocking sites on platelets for fibrinogen binding.

Factors regulating PLA<sub>2</sub> are also being studied to understand many other as yet unclarified cellular events. Recently, this group has described two nonapeptides, derived from uteroglobin and lipocortin I respectively, which are potent inhibitors of PLA<sub>2</sub> *in vitro*. A novel mechanism of activation of PLA<sub>2</sub> by tissue and plasma transglutaminase (TG) has been identified. Members of the group found that: a) both plasma and tissue (guinea pig liver) TG activate porcine pancreatic PLA<sub>2</sub> *in vitro*; b) this activation is both time and dose dependent; and c) the addition of polyamine substrates of TG in micromolar concentrations significantly reduces the activation. Maximal stimulation (PLA<sub>2</sub> activity 400% of control) is obtained at concentrations of 9 x 10<sup>-2</sup> units/ml of guinea pig liver TG and with 200 µg/ml of rabbit plasma TG (coagulation factor XIII, preactivated with thrombin). Time courses of the PLA<sub>2</sub> reaction, analyzed by the integrated form of the Michaelis-Menten equation, show that the apparent K<sub>m</sub> of PLA<sub>2</sub> is not changed, while the apparent V<sub>max</sub> is increased by TG treatment. Since it is known that dimerization and aggregation of PLA<sub>2</sub> result in increased enzymatic activity, the action of TG may represent a novel mechanism, by which activated forms of PLA<sub>2</sub> can be systematically stabilized. These data suggest that a novel post-translational modification of PLA<sub>2</sub> could play a role in pathological processes, such as inflammation, thrombosis and disseminated intravascular coagulation. A collaborative clinical study to evaluate arachidonic acid metabolism in cystic fibrosis is in progress, and the investigations on a possible genetic defect in fetal alcohol syndrome is also being continued.

The Section on Drug Biotransformation, under the direction of Ida Owens, studies the regulation of the UDP-glucuronosyltransferase gene family at the molecular level using both mouse and human systems. Since an undetermined number of transferase isozymes is involved in detoxifying numerous endogenous and exogenous lipophiles through glucuronidation, the aim of this research program is to isolate and characterize the cDNAs encoding the many isozyme activities, including the one for the critical metabolite, bilirubin. A mouse cDNA, UDPGT m-1, has been characterized and shown to encode a protein of 51 kDa, which is glycosylated through an asparagine linkage. This isozyme catalyzes a broad range of substrates, when expressed in yeast *Saccharomyces cerevisiae* (AH<sub>22</sub>) using the vector pEVPII. The substrates include three androgens, an estrogen, three aromatic compounds and two with both aromatic and saturated-ring structures. When the nucleotide sequences encoding the membrane-targeting signal peptide were removed and the 5' end was adapted with a new AUG translation-start site, the protein was located in the cytosol rather than the membrane. The catalytic activity of the cytosolic form is very much reduced for all substrates except naphthol, which is the preferred substrate of the membrane-bound version of the enzyme.

Using UDPGT m-1 as a probe, a human liver cDNA, UDPGT h-1 has been isolated and characterized. It is 2000-bp in length and is encoded in three different sized mRNAs, 2.3 kb, 3.6 kb, and 5.3 kb, attributable to corresponding increments in lengths of 3' untranslated extensions. The 2.3 kb species is highly abundant, while the 5.3 kb species is barely detectable. When inserted into pEVPII and expressed in AH<sub>22</sub> cells, the cDNA encodes a 51-52 kDa protein, which fails to catalyze any of 50 potential substrates tested. (Further studies on substrate activity are continuing in Hep.G2 and COS-1 cells using the pSVL expression

vector.) UDPGT h-1 shows a *Stu*I-polymorphism, in which both extra 3.2 kb and 4.3 kb DNA fragments can be demonstrated by Southern blot analysis. This genotype appeared in 24 of 27 members of the American population (not including Orientals). A screen of DNA from 18 individuals showed that 0/3 Koreans, 1/7 Chinese and 2/8 Japanese contained the extra fragments. Finally, by using UDPGT h-1 as a probe, Owens' group uncovered for the first time a human liver "catechol" estrogen UDP-glucuronosyltransferase clone, UDPGT h-2. The clone is encoded in a 2.1 kb to 2.2 kb mRNA species. Expression of the insert, contained in a pSVL vector, in COS-1 cells reveals that a 51 kDa glycosylated protein is synthesized. The preferred substrate activity of the isozyme is as follows: 4-OH estrone > 2-OH estriol > 2-OH estradiol = 6 $\alpha$ -OH estradiol > estriol > 4-OH estradiol > 2-OH estrone. It does not glucuronidate the estrogens ( $\beta$ -estradiol or estrone), 16 $\beta$ -estrone, 16 $\alpha$ -OH estrone, 16 $\alpha$ -OH progesterone, 16 $\alpha$ -OH pregnenolone, testosterone, dihydrotestosterone, androsterone, 16 $\alpha$ -OH testosterone, the catecholamines or some 50 other potential substrates tested. The existence of an isozyme, which glucuronidates a comparatively narrow substrate group, including the catechol estrogens, could be crucial in alleviating the inhibitory effect of these estrogen derivatives on the catechol amine-O-methyltransferase enzyme.

Samuel Adeniyi-Jones has demonstrated that secondary structures of RNA affect the function of Alu RNA transcripts in the cell. The alpha fetoprotein  $\beta$ -1 Alu sequence forms an identical secondary structure with the Alu portion of 7S RNA. It can, therefore, bind to the same proteins as 7S RNA. Differences of a few nucleotides in Alu sequences change the secondary structure of RNAs. Since these can have a differential affect on translation, this offers evidence for a role of these RNAs in the regulation of translation. The group has also purified the protein  $\alpha$ -63 to approximately 80% homogeneity. This protein displays a tissue-specific and developmental pattern of expression in *Xenopus*. The protein binds primary and processed transcripts of injected repeat sequences in the nucleus and cytoplasm.

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## CELL BIOLOGY AND METABOLISM BRANCH

Richard D. Klausner, M.D., Chief



Left to right: W. Leonard, J. Harford, R. Klausner, L. Samelson

The Cell Biology and Metabolism Branch (CBMB) has continued its work in the areas of iron metabolism, gene regulation, immunology and cell biology. The work of the laboratory is divided into several projects, involving the cellular and molecular biology of human iron metabolism, biochemical aspects of T cell activation, the structure, biosynthesis and cell biology of T cell antigen and interleukin 2 receptors, and the cell biology of the secretory pathway. This past year has been an extremely productive one for the CBMB. This productivity is attested to by the more than 55 publications produced over the past 12 months. The laboratory has maintained its clinical activity in studying the problems of diseases of human metal metabolism. In addition, the laboratory has continued its interest in the molecular and cell biology of the human immunodeficiency virus (HIV), the etiologic agent responsible for AIDS.

The long term interest of this laboratory in understanding the basis of iron metabolism is pursued by two groups; one is led by **Joe Harford**, which studies the transferrin receptor (TfR), and the other is under the direction of **Richard Klausner**, which studies the regulation of intracellular iron metabolism. The importance of iron metabolism in human biology is apparent in many areas of research. Because iron is absolutely essential for and likely to be rate-limiting for cell growth, the ability of cells to regulate and assure iron uptake is strictly correlated with their proliferative rate. This is true during development, in normal differentiation, proliferation, inflammation, the immune response, wound healing, and in neoplasia. In addition to its correlation with cellular growth, iron is absolutely required for the maintenance of baseline metabolic activities. Thus, obtaining sufficient iron is critical for the health of both the individual cell and the entire organism. This importance is reflected in the wide range of clinical consequences of iron deficiency, a common clinical disorder. In contrast to iron deficiency, where not enough iron leads to pathologic

consequences, too much iron is extremely toxic and eventually leads to cell death. The inability to regulate the uptake of iron underlies one of the most common genetic diseases of man, hereditary hemochromatosis. This disease, which affects one in 400 people in our population, is due to a failure to regulate iron uptake, and results in a high rate of morbidity and, if untreated, mortality. The CBMB is at the forefront of studies aimed at elucidating the molecular basis of iron metabolism. Studies by the Branch continue to focus on how genes are regulated by iron. This aspect of iron metabolism is being emphasized because genetic regulation underlies cellular mechanisms that attempt to prevent both iron deficiency and iron toxicity. The work on the regulation of proteins involved in human iron metabolism has not only elucidated that specific problem but has also uncovered a major new area of post-transcriptional gene regulation. The identification of specific *cis*-acting RNA elements and regulatory *trans*-acting RNA binding proteins has placed post-transcriptional gene regulation on a firm and detailed molecular basis. We now know that there are two critical elements that underlie the iron regulatory system: the transferrin receptor, along with its ligand, transferrin, and ferritin. Work from this laboratory has brought us very close to a molecular understanding of how these genes are regulated in response to iron. Ferritin is regulated at the translational level, such that when there is little iron there is very little translation, and when there is a large amount of iron there is a larger amount of translation. The transferrin receptor is predominantly regulated at the level of the half-life of the mRNA encoding the protein. In contrast to ferritin, when there is little iron, mRNA levels for the transferrin receptor increase, due to a longer half-life, and when there is more iron the half life of the mRNA shortens, resulting in a drop in receptor number and less iron uptake into the cell.

Work in this laboratory over the past several years has allowed two molecular elements to be defined that underly the regulation of these two genes. One is an RNA element, a sequence found within the messenger RNA molecules encoding both ferritin and the transferrin receptor. This element, which Klausner and his colleagues refer to as an iron-responsive element or IRE, is a small stem-loop structure, which endows iron sensitivity to the regulation of the fate of the RNA. A single IRE present in the 5' untranslated region (5' UTR) of ferritin mRNA molecules is entirely responsible for the ability of iron to regulate the translation of the ferritin message. The domain of the mRNA of the transferrin receptor regulating its iron responsiveness is located within the 3' untranslated region. This region has been shown to contain 5 RNA motifs with sequences resembling the ferritin IRE.

The second molecular element involved in the regulation and expression of these iron related genes is a cytosolic protein (proteins), which specifically binds with high affinity to the IRE. This protein has been identified by the use of a combination band shift and RNase protection assay. As predicted from sequence analysis, the same cytosolic binding protein interacts with the IRE found in the ferritin 5' UTR and with IREs identified in the regulatory region of the 3' UTR of the transferrin receptor. These observations have allowed the group to formulate a unifying model to explain the apparent disparate regulation of these two genes. According to this model, when there is little iron around, the IRE binding protein (IRE-BP) becomes activated and binds to the IRE. If the IRE is in the 5' UTR of the gene, the binding of that protein will sterically block the initiation of translation. For the transferrin receptor mRNA, the situation is more complex. Message half-life is regulated by iron via the IREs and their interaction with the binding protein, i.e., the absence of iron activates the IRE-BP to bind to the RNA regulatory element. Evidence for the existence of two functional elements in the regulatory region of the TfR mRNA has been obtained. The first endows the region with with iron responsiveness and can be entirely ascribed to the ability to bind to the IRE-BP. The second is a functional element, which has no bearing on the interaction with the iron dependent *trans* factor, but is an mRNA instability determinant. It is clear that these two elements are intimately intertwined structurally, and it is the dissection of these two elements that allows us to propose a model for this regulated stability. In the absence of iron, the IREs in the regulatory region are bound by the IRE-BP, and this blocks the function of the intrinsic RNA instability element. Thus, in the absence of iron, the mRNA is stabilized. In this way, the increased binding of the IRE-BP in the absence of iron can explain both the decreased rate of biosynthesis of ferritin and the

increased rate of biosynthesis of the transferrin receptor. Support for this model has been provided by the finding that the specific activity of the IRE-BP in the cytosol is considerably increased when cells are treated with an iron chelator in order to starve them of accessible iron. In addition to this model for how the IRE-BP could regulate iron-responsive genes at the post-transcription level, the CBMB is beginning to unravel the actual biochemical mechanisms by which the IRE-BP may both respond to iron levels and interact with the IRE.

Major advances in our understanding of the IRE-BP have accrued over the past year. First of all, the IRE-BP has been purified to homogeneity, using a novel RNA affinity purification scheme. The protein has been purified in significant amounts, and has been subjected to extensive peptide amino acid sequencing. Using the extensive protein sequence now available, attempts are being made to clone the cDNA encoding this protein. It is a relatively abundant 90 kD protein. In addition, the group has established what it believes to be a novel biochemical regulatory mechanism to explain the iron sensitivity of the interaction of the IRE-BP with its cognate RNA elements. The IRE-BP was found to exist in two binding states, a low affinity state ( $K_D = 5 \text{ nM}$ ) and a high affinity state ( $K_D = 10\text{--}30 \text{ pM}$ ). In the absence of iron, a large fraction of the total population is present in the high affinity state. Conversely, in the presence of iron, the vast majority (greater than 99%) is found only in the low affinity binding state. The switch between the two affinity states appears to be a reversible redox reaction within the protein. Thus, at least one critical pair of sulfhydryls, when oxidized, causes the protein to assume the low affinity configuration. When this disulfide bridge is reduced to produce free sulfhydryls, the protein is in the high affinity binding state. These two redox states of the protein are determined within the cell by changes in iron status, and are each quite stable. This is the first known example of a cytosolic protein being regulated by this type of "sulfhydryl switch."

In addition to the TfR, transferrin, and ferritin, other critical genes are undoubtedly involved in human cellular and total body iron metabolism. However, the identification of these genes is a major problem. Because the essentials of iron metabolism are likely to be similar in all organisms, be they primitive prokaryotes, yeast, or higher eukaryotic mammalian cells and organisms, members of the CBMB are examining iron metabolism in the yeast *Saccharomyces cerevisiae*. They have shown that this organism is absolutely dependent on the uptake of environmental iron for health, growth and proliferation. They are using both biochemical, protein chemical and, most importantly, genetic approaches to identify genes and gene products of this yeast that are involved in iron uptake. In particular, an iron reductase gene has been identified in the membrane of yeast cells, which is responsible for the initial events in the transport of iron across cellular membranes. This ferric reductase gene has now been isolated and cloned, and shown to be highly regulated in response to a variety of environmental changes, including nutrient deprivation, oxygen status and, most importantly, iron status. Transmembrane uptake of iron is one of the major gaps in our understanding of human iron metabolism. Obtaining the yeast gene encoding this iron reductase should allow the corresponding higher eukaryotic gene to be identified.

One of the goals of all of these studies is the application of the insights gained to diseases of iron metabolism. Thus, the Branch maintains a very active clinic, in which patients with hereditary hemochromatosis are examined, and continuous cell lines established from their peripheral blood lymphocytes. This provides not only cells from these patients, with which to study physiologic aberrations, but also a source of genetic material for investigations into the molecular basis of possible defects underlying hereditary hemochromatosis. In addition, these studies are yielding unique insights into specific mechanisms of gene regulation in human cells. They are also providing useful applications of recombinant DNA technology. Accordingly, the discovery of the IRE has led to the patenting of this new type of genetic element for possible use as part of an iron-regulated expression vector system.

Largely due to the work of this laboratory, the T cell antigen receptor (TCAR) is now understood as one of the most complex integral membrane receptor molecules. This complexity applies both to subunit structure and biochemical function. We now know that the receptor is composed of at least seven different proteins, which are assembled in

complexes consisting of seven, nine or more chains. **Richard Klausner** (Klausner studies the structure, biosynthesis and cell biology of the TCAR) and **Lawrence Samelson** (Samelson is concerned with biochemical basis of T cell activation), who direct different aspects of this project, who have called the two most recently defined chains of this receptor complex zeta and eta. Both the murine and the human zeta chain genes have been cloned by these investigators in the past year, and this has led to a complete elucidation of the structure of the protein. The zeta chain occurs in two different forms, as a homodimer and as a heterodimer. As a heterodimer it is linked to the somewhat larger eta chain. Zeta is about ten times more abundant than eta, and thus the heterodimer is most probably only found in the minority of surface receptors. One of the goals of these studies is to correlate the structure of the receptor with its function. Receptor function is defined by examining both the proximal biochemical events, which ensue upon receptor stimulation, or the resulting phenotypic changes in cells (generally referred to as cell activation). In order to make these structure/function correlations, two approaches are taken: 1) isolation of mutants or variants of antigen specific T cell hybridomas; and 2) reintroduction of genetically altered TCAR subunits into deficient T cell lines. Over the past year much progress has been made using both of these approaches. In particular, functional consequences of failing to synthesize either the zeta or the eta chain have been examined. The absence of the eta chain has no effect on the surface expression of the receptor complex. However, it does seem to be correlated with severe functional deficiency. This group has previously shown that the TCAR functionally couples to at least two cellular biochemical pathways. In one, phosphorylated phosphatidylinositides are broken down in response to receptor activation, leading to the release of water soluble inositol phosphates and diacylglycerol. The result of this is the activation of protein kinase C and the mobilization of intracellular calcium. The other pathway involves the activation of a non-receptor tyrosine kinase or tyrosine kinases, which result in the tyrosine phosphorylation of a number of cellular substrates. In the absence of eta, receptor mediated tyrosine kinase activation is maintained, while the ability to couple the phosphatidylinositide (PI) pathway is lost. A variety of eta-negative cell lines have been established, which have allowed the group to solidify its model for two functional classes of T cell receptors, one containing zeta-zeta, and one containing zeta-eta. As previously noted, zeta-eta containing receptors are responsible for coupling to PI metabolism. Interestingly, cells that have no eta are capable of making normal amounts of interleukin (IL-2). In contrast, cells that have no eta are not capable of undergoing receptor-mediated cell death. This is particularly intriguing, if one assumes that cell mediated apoptosis is currently the best model for negative selection in the thymus. The finding that PI turnover is not necessary for IL-2 production raises the question of whether the tyrosine kinase pathway is capable of stimulating IL-2 production. One approach to testing this was to isolate stable transfectants of 2B4 T cell hybridomas containing and expressing the *v-src* oncogene, an active tyrosine kinase. When this happens, a variety of intracellular substrates, normally phosphorylated in response to TCAR engagement, are constitutively phosphorylated. More importantly, these cells also produce IL-2 constitutively. When the rate of transcription of the transfected gene is increased, the rate of production of IL-2 is likewise increased. This is the first demonstration of the ability to mimic a particular cellular activation pathway by the introduction of an exogenous unregulated tyrosine kinase.

Structure/function correlations for the TCAR involve the characterization of the eta chain and mutagenesis of the zeta chain, followed by reconstitution of zeta deficient T cell clones. The eta chain has been extensively characterised biochemically, and antibodies that recognize both the human and murine eta chain have been raised. In addition, the zeta chain has been subjected to site-directed mutagenesis, followed by reintroduction of the mutated zeta chain to reconstitute zeta deficient T cells. This has resulted in the demonstration that truncation of the cytoplasmic tail of zeta essentially destroys the information to flow from the alpha-beta ligand recognition chains to the coupling CD3 chains. These studies represent the first attempts to dissect components involved in information flow within this complex signalling structure on a molecular level.

As stated above, multiple kinases are activated in response to the stimulation of the T cell antigen receptor. It is the belief of this group that understanding the nature of these kinases,

their pattern of activation and regulation, their interaction, and relevant cellular substrates is absolutely critical to our ability to understand and possibly manipulate the immune response. A set of cellular substrates that are tyrosine phosphorylated in response to receptor activation have been defined. Recent data suggests that there may be two different sets of substrates phosphorylated in response to receptor occupancy, and that these two sets may be the targets of two different tyrosine kinases. One set includes the rapid phosphorylation (within seconds) of a cytosolic protein called pp62. After pp62 is phosphorylated, the zeta chain of the T cell receptor is phosphorylated. Another set of substrates has been defined, which appear to be extremely susceptible to dephosphorylation by a tyrosine phosphatase within the cell. In order to see the phosphorylation of this set of substrates, it is necessary to examine the cell in the presence of the tyrosine phosphatase inhibitor. The pharmacologic characteristics of the phosphorylation of these two sets of substrates are quite distinct. Attempts are currently being made to purify the pp62 tyrosine kinase substrate, as it may be the tyrosine kinase linked to the TCAR. Changes in the major T cell membrane tyrosine phosphatase, CD45, in response to receptor activation were also examined. In the course of these studies, a novel and intriguing cellular activation pathway has been defined, which changes the intracellular distribution of CD45 in response to receptor activation. This represents the first example of a change in the residency time of a protein in an intracellular organelle in response to external signalling. The role of this redistribution in T cell activation is currently being examined. In response to receptor stimulation, an additional cytosolic kinase is phosphorylated. This is the cellular homolog of the *raf*-oncogene. This kinase is a serine/threonine specific kinase, and every molecule of *c-raf* within the T cell is phosphorylated, perhaps on multiple sites, in response to receptor activation. The regulatory consequences of the phosphorylation of this proto-oncogene product are being explored.

T cell activation is accompanied by a genetic program whereby a number of genes are turned on in a predictable and defined pattern. This pattern of genetic program expression is stereotyped both in terms of kinetics, order of genes, and the groups of genes involved. One of the most important sets of genes that are turned on involve the interleukin-2 system, which is being studied by a group led by Warren Leonard. Interleukin-2, or T cell growth factor, provides autocrine, paracrine and endocrine stimulation for the proliferation of T cells. For T cells to respond to this growth factor, they must express a receptor that is specific for it. The IL-2 receptor group has continued to examine several aspects of its biology. They have directed their attention towards determining the genetic elements involved in the carefully regulated expression of the interleukin-2 receptor alpha subunit gene. Using a variety of techniques to study transcriptional elements in the 5' genomic flanking region, as well as techniques aimed at looking at specific DNA protein interactions, this group has come a long way in elucidating the sequences involved in the expression of this gene. In addition to the regulated expression of this gene during the activation of T cells, the gene for the IL-2 receptor alpha chain is also highly expressed in an apparently unregulated fashion in cells infected with, and transformed by, the human T cell leukemia virus HTLV-I. This group of workers is defining those elements that appear to be responsive to the expression of gene products encoded by this virus. One area, which has received considerable attention over the past year, is the potential role of a particular DNA binding protein, first described as an enhancer element for the kappa immunoglobulin light chain gene. A consensus sequence that defines this element binds a particular transcription factor, called NF-kappa-B. It appears that the same motif is present and capable of binding an NF-kappa-B molecule (or molecule closely related to it) in the regulatory region of the IL-2 receptor gene. The role of this transcriptional factor in the expression of the IL-2 receptor alpha chain gene is being explored. This is of particular interest because of the role of these sequences and this factor in the activation of the human immunodeficiency virus HIV. In addition to the NF-kappa-B binding site, a variety of other protein binding sites have been identified within this small region critical to the expression of the IL2R-alpha gene. These include the previously described serum response factor SP1, and one or two newly defined binding proteins.

Another aspect of the biology of the interleukin-2 receptor explored by this group is the role of the beta subunit, called p70, first described by this group two years ago. The ability of

the beta subunit to exist on the cell surface in the absence of the alpha subunit, and to serve as a receptor in these cells has been demonstrated. This is true of a variety of peripheral blood mononuclear cells, such as large granular lymphocytes and the precursors of LAK cells and NK cells. That the beta subunit can be a receptor, or part of a receptor, in the absence of the alpha subunit is demonstrated both by its ability to bind IL-2 and to mediate biologic responses to that binding. The beta chain of the IL-2 receptor is not restricted to T cells, but can be induced on the surface of both peripheral blood B cells and monocytes. The potential roles in immunobiology of this receptor subunit are only beginning to be explored. The recent cDNA cloning of the beta chain of the IL-2 receptor has allowed this group to isolate full-length cDNAs, as well as genomic clones, which are being analyzed for critical regulatory elements.

Finally, this group has been engaged in characterizing other genes that are rapidly turned on in response to T cell activation. One of these, which has been referred to as Act-II, is rapidly turned on in both T cells and other mononuclear cells in response to stimulation. The gene and cDNA encoding this protein have been characterized. The cDNA predicts a small secreted protein, suggesting that this may be a new lymphokine. In addition, this protein has recently been expressed in a variety of systems, including a baculovirus expression system in cultured insect cells. This system secretes a large amount of the protein, which will allow more biochemical analysis, as well as functional studies of this novel T cell activation gene product.

Work in the laboratory in the area of cell organelle biology has exploded over the past year, necessitating the formation of a new project, which is led by **Richard Klausner**. Much of this work stemmed from initial studies on the problem of the assembly and intracellular transport of the seven membered TCAR complex. These studies led to the formulation of the concept of "architectural editing," an overall set of cellular processes that relate the fate of newly synthesized multicomponent complexes within the cell to the structure of those complexes. The group has been examining the signals used in architectural editing, the intracellular pathways by which incorrect complexes are edited out of the cell, and the recent finding that architectural editing is not simply a mechanism of quality control for abnormal proteins, but also a mechanism for the regulated expression of a variety of protein complexes by the cell. Because so much of architectural editing takes place within the endoplasmic reticulum (ER), the group's attention has been focused on new structures and functions within that complex organelle.

In last year's Annual Report, a new cellular pathway was described, which is responsible for the degradation of newly synthesized proteins that are deemed to be structurally abnormal by the cell. This year, attention was focused on trying to understand one of the most striking aspects of ER degradation—its specificity. Initially, this was identified by studying the fate of subunits of the seven-membered T cell antigen receptor when retained within the endoplasmic reticulum. It was found that three of the subunits are selectively and rapidly degraded by this non-lysosomal degradative system, while the remaining subunits are stable for long periods of time within the organelle. The degraded subunits include the alpha chain, the beta chain and the delta chain, while the gamma, epsilon and zeta chains are stable. One observation, which may be pertinent to this selectivity, is that if the complex is prevented from leaving the ER, it begins to disassemble. This disassembly can be blocked by relatively minor reductions in the temperature. Interestingly, when the temperature is reduced to prevent disassembly, no degradation is seen. The degradation itself appears to be temperature sensitive, and all ER degradation is blocked at temperatures below about 15°C. Thus, if the alpha chain or alpha-beta heterodimer are expressed in fibroblasts in the absence of any other subunits, ER degradation takes place at all temperatures above 16°C, at which point there is an apparent phase transition in the activation energy for degradation. However, the temperature coefficient for ER degradation of assembled alpha-beta in T cells changes dramatically, there is no longer a phase transition, and the degradation is dramatically inhibited by simply dropping the temperature to 30°C. The relationship between assembly and degradation within the ER was further examined by attempting to answer the question why the CD3-gamma chain is not degraded. This is puzzling, because CD3-gamma is so

similar to the CD3-delta, which is degraded. To test this, the cDNA encoding CD3-gamma was transfected into fibroblasts. In this case, gamma does not leave the ER and is rapidly degraded by the ER. In contrast, if the epsilon chain is co-transfected, epsilon-gamma complexes are formed, and this prevents the degradation of gamma. When epsilon is transfected alone, it is not subject to ER degradation.

A more definitive approach to the selectivity of degradation has been to dissect the alpha chain of the T cell receptor and to ask the question whether any region of this protein contains the information for ER degradation. When the transmembrane domain and five amino acid cytoplasmic tail are removed from alpha, leaving a truncated, potentially secreted protein, this truncated protein, when expressed in cos cells, is not secreted, remains in the ER, but is not rapidly degraded. To test whether the relatively short sequence removed in this experiment contains information to target other proteins for ER degradation, the transmembrane and cytoplasmic domains of alpha were fused with the luminal domain of the IL-2 receptor Tac antigen. The IL-2 receptor, when expressed in cos cells, is not degraded, even if it is forced to remain in the ER by treatment with the drug Brefeldin A. Furthermore, the truncated form is rapidly secreted and again, if it is retained in the ER by treatment with this drug, there is no ER degradation. However, when the chimeric protein is made, it is both retained in the ER and targeted for extremely rapid ER degradation.

Another part of this project concerns membrane dynamics in the secretory system. In looking for pharmacologic reagents that might block functions associated with the endoplasmic reticulum, studies reported by a group of Japanese researchers on the fungal antimicrobial agent Brefeldin A were noted. This is a prostaglandin-like, heterocyclic lactone, made by a variety of fungi. It had been reported to block secretion out of the ER. The group began examining its effect on the cell, and made a remarkable finding. When cells are treated with this drug, the Golgi begins to disassemble within minutes, and by ten minutes integral membrane proteins of the Golgi are found uniformly through the endoplasmic reticulum. This is the first observation of the ability to specifically return Golgi proteins to the ER. The ER now functions as a mixed ER and Golgi, and carbohydrate side chains of glycoproteins retained in this organelle are now processed as if they have reached the Golgi. The possible presence of a retrograde pathway within the secretory system challenges many of our notions of the structure, function, and dynamics of the secretory pathway. This retrograde movement induced by Brefeldin A has been more carefully studied. It is a temperature sensitive process, which seems to be divided into two discrete steps. At temperatures below 16°C Brefeldin A has no effect on the Golgi. At temperatures between approximately 18-22°C the Golgi appears to fuse into a large vacuolar structure, which gives off long strings of necklace-like tubulo-vesicular extensions. However, at this intermediate temperature no apparent fusion with the ER is observed. At temperatures above 22°C the Golgi distributes through the ER. A striking observation was that agents that disrupt microtubule structure or function, such as microtubule depolymerizing agents (nocodazole) or others (griseofulvin), block the movement of the Golgi back to the ER. When cells are pretreated with nocodazole and then given Brefeldin A, the Golgi seems to move into large scattered structures that, interestingly, co-localize with antibodies directed against transition vesicles, organelles in the secretory pathway that are believed to exist between the ER and the Golgi. In contrast to this retrograde movement, anterograde movement out of the ER and back into the Golgi is unaffected by the agents that disrupt microtubule function. The next question was whether nocodazole would block a recycling pathway that operates either between the cis-Golgi or pre-Golgi compartment and the endoplasmic reticulum. Remarkably, proteins that are retained in the ER, either due to treatment with Brefeldin A or because of the presence of the KDEL "ER retention" signal, rapidly accumulate in this intermediate compartment upon treatment with nocodazole. This is the first evidence for the structure and identity of an ER recycling/retrieval pathway.

The ability to completely block movement out of the endoplasmic reticulum was used to test whether the ER is involved in the presentation of antigens. Initially, the presentation of an endogenous antigen, an internal peptide of the influenza virus matrix protein, by class I major histocompatibility complex (MHC) molecules was examined. This was done using a

human B cell line as a target for cloned human T cell killer lines. It was found that Brefeldin A, while not blocking viral protein synthesis, completely blocks the ability to present endogenous antigens by the class I pathway. It does not block the ability of these same cells to present the processed peptide, when the peptide is added to the outside of the cell. In addition, it does not affect the ability of class II MHC molecules to present their peptides, and nor does it block the ability of antigen presenting cells to process exogenous antigens presented by class II molecules. However, the group recently found that the ability to present endogenous antigens by class II molecules is also blocked by Brefeldin A, as is the class I endogenous pathway. This work has allowed the proposal of a new model for the cell biology of antigen presentation, and of pharmacologic agents blocking this process.

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## LABORATORY OF DEVELOPMENTAL AND MOLECULAR IMMUNITY

John B. Robbins, M.D., Chief



Left to right: J. Robbins, R. Schneerson, S. Szu, K. Ozato

The Section on Bacterial Disease Pathogenesis and Immunity, under **John Robbins**, is concerned with pathogenesis and natural and acquired immunity to invasive diseases, especially in neonates and young children. Robbins and his colleagues **Rachel Schneerson** and **Shousun Szu** are working on the problem of the synthesis and protective actions of conjugate vaccines composed of capsular polysaccharides and de-lipidated LPS. The stimulus for this work derives from the fact that, in children, T-dependent properties of the *Haemophilus influenzae* type b capsular polysaccharide (Hib), and other of polysaccharides of invasive bacteria, only develop with age, which limits their protective actions in infants and children, the age group with the highest attack rate of diseases due to these pathogens. The envelope polysaccharides of some other pathogens are either not or only poorly immunogenic, even in adults. Several years ago, therefore, an organic synthetic scheme by which Hib and other capsular polysaccharides are bound to carrier proteins, in a clinically acceptable manner, was devised to both increase the immunogenicity of and confer T-cell dependence (booster effect) on these protective antigens. A conjugated Hib vaccine, prepared by the original method of the LDMI, was licensed by the FDA for universal use in children older than 18 months of age. The safety and immunologic properties of our Hib-TT vaccine was recently investigated in 18-23 month olds and is now being examined in 2-3 month old infants. One injection of Hib-TT into 18-23 month old children elicits protective levels of antibodies in all recipients. One year following the injection, these antibodies were statistically higher than the age-related controls. In clinical trials conducted in Goteborg, Sweden, and in Charlotte, N.C., three injections of Hib-TT to infants showed that protective levels are reached in almost all subjects after two injections and in all after three injections. Neutralizing antibodies to tetanus toxoid, the carrier protein, are also elicited by these vaccines. It is estimated that preventing Hib infection in infants and toddlers with this new vaccine will reduce the incidence of meningitis in this previously

unprotected group by at least half and thus eliminate the most common cause of acquired mental retardation in this country.

To allow conjugation of a wide range of other capsular polysaccharides, the efficacies of various types of joining reagents were compared. Polysaccharides having carboxyl groups, single hydroxyl, or vicinal hydroxyl groups each require different cross-linking reagents for conjugation with carrier proteins. Bacterial polysaccharides tested were: Vi, *pneumococcus* types 6B, 12F, 19F, 19A, and 23F, O-specific side-chains of *Shigella dysenteriae* type 1, *Salmonella paratyphi* A, *Staphylococcus aureus* types 5 and 8. The Vi was modified by O-deacetylation and decarboxylation in order to study the relationship between structure and immunogenic epitopes. The immunogenicity of Vi conjugates, prepared with polysaccharides of either high or low molecular weights, with the  $\beta$ -subunit or holotoxin of cholera toxin were compared in laboratory animals. It was found that the carboxyl group is not important for immunogenicity, whereas O-deacetylation reduces immunogenicity. The technology was used prepare conjugates of *Pneumococcus* type 6 bound to tetanus toxoid. This *pneumococcus* conjugate vaccine elicited protective levels of antibodies in 96% of 2-5 year-old sickle cell anemia patients, whereas none of the age-matched patients injected with the unconjugated *Pneumococcus* type 6 polysaccharide showed protective levels. Similar results have been obtained with a tetanus toxoid conjugate of *Pneumococcus* type 12F in human volunteers and, in mice, with a pertussis toxoid conjugate of *Pneumococcus* type 14 (the most common cause of otitis media), and with a tetanus toxoid conjugate of the closely related group B *streptococcus* (which causes neonatal sepsis). New methods to synthesize conjugates for the unusual capsular polysaccharides of group B *meningococcus* and *E. coli* K1, which in themselves are not immunogenic, are under study.

Enteric fevers remain a serious and frequent cause of morbidity and mortality throughout the world. Development of effective vaccines for the prevention of these diseases would have a major impact on public health. These intestinal diseases are caused by pathogens of the genus *Salmonellae*. The epidemiology and frequency of enteric fevers differ throughout the world. The most frequent and serious type in underdeveloped nations is typhoid fever caused by *Salmonella typhi*, followed by *S. paratyphi* A. In this country the most frequent species encountered are *S. typhimurium* and *S. enteridis*. Because most *Salmonellae* are inhabitants and pathogens of humans only, evaluation of vaccines for the prevention of these diseases has a long and variegated history. The currently licensed cellular typhoid vaccine confers immunity for only a limited time and has a high rate of adverse reactions. A few years ago workers in the LDMI prepared a typhoid vaccine consisting of the Vi capsular polysaccharide, which was able to protect 70% of the population in parts of the world that have a high attack rate; but protection rates have declined in the three years since the trial was conducted. To improve the immunogenicity of this vaccine, conjugates were prepared by covalently binding the polysaccharide to either tetanus toxoid, cholera toxin or its nontoxic  $\beta$ -subunit. Clinical studies of a Vi-tetanus toxoid conjugate have been started.

The surface carbohydrates of other *Salmonellae* and *Shigellae* are not capsular polysaccharides but lipopolysaccharides (LPS). The lipid A component of LPS, which confers disease symptoms, has to be removed before the polysaccharide can be considered for immunization. This lipid-free moiety is too small to be used alone as an antigen and must therefore be conjugated to carrier proteins to make suitable vaccines. Carrier proteins being tested include the  $\beta$ -subunit of cholera toxin, the heat-labile enterotoxin of *E. coli* and the enterotoxin of *Shigella*.

The incidence and severity of pertussis (whooping cough) have been controlled by immunization with inactivated *Bordetella pertussis* organisms (cellular vaccine) as the P component of DTP. The identification of pertussis toxin as a major, if not the sole, protective antigen for *B. pertussis* has led to the development of a toxoid derivative of this antigen. Pertussis toxin was purified by affinity chromatography from the culture supernatant of *B. pertussis* and inactivated by hydrogen peroxide under controlled conditions. The resultant product, called NICHD-PTxD, had less than 1% of its original binding and enzymatic activities and shows no pharmacologic properties of the holotoxin *in vivo*, by such

assays as the histamine sensitization test. Clinical evaluation of the first investigational lot of this toxoid vaccine in adults and in 18-month old children have shown it to be safe and more immunogenic than the cellular pertussis vaccine component. Antibodies induced by the toxoid neutralize the biologic properties of the holotoxin (antitoxin). The toxoid-induced IgG antibodies are almost exclusively of the IgG1 subclass, with a small percentage in the IgG4 subclass. A clinical evaluation of the second investigational lot of the toxoid in Swedish infants, injected at either three, five and seven, or three, five and twelve months according to the recommended schedule, showed no local or systemic reactions, confirming its superior safety characteristics. The serological response to this investigational vaccine is now under study.

The Section on Molecular Genetics of Immunity, under **Keiko Ozato**, is studying the mechanisms of gene regulation in the developing immune system with a major emphasis on transcriptional control of the MHC class I genes. MHC class I genes encode a series of polymorphic transplantation antigens, which regulate the development and functions of the immune system. In previous studies, Ozato and her colleagues identified a complex in the 5' upstream region, which contain two regulatory elements capable of controlling transcription of MHC class I genes. One of these sequences is the conserved *cis*-acting class I regulatory element (CRE). The CRE is juxtaposed to another regulatory element, the interferon consensus sequence (ICS), which is responsible for interferon (IFN)-mediated induction. By analogy with other regulatory systems, the CRE and the ICS would be expected to control transcription by interacting with *trans*-acting nuclear proteins. Two separate nuclear factors were identified that bind to different regions of the CRE (the CRE contains at least three discrete sequences, regions I, II, and III), and which enhance the transcription of the MHC genes. Another factor was found that is induced by IFN and is necessary for IFN dependent transcription of the MHC genes. MHC transcription is developmentally controlled, expression remaining very low during gestation, but increasing sharply after birth. The repression of MHC genes in early embryonic cells is also governed by the CRE-ICS complex. A nuclear factor that binds to a negative regulatory site in the complex has been detected in embryonal carcinoma cells, but not in other cells that do not exhibit the MHC gene repression. These findings suggest that factor binding to the conserved CRE-ICS complex is the control mechanism that governs transcription of the MHC genes. The genes encoding these *trans*-acting regulatory factors are being sought by screening phage expression libraries, using CRE and ICS as probes. This method relies on *in situ* DNA-protein interactions to isolate factor cDNA, and does not require detailed information on the protein structures. Two cDNA clones have been isolated that encode proteins capable of binding to the MHC regulatory complex. These clones, called ICSB and H-2RIIBP, bind to the ICS and to region II of the CRE respectively. H-2RIIBP is composed of a zinc finger and modular domains characteristic of the steroid/thyroid hormone receptors, and was found to be capable of binding to the estrogen response element of the vitellogenin gene. H-2RIIBP is expressed widely in mouse tissues, and is conserved among many animal species, including *Drosophila*. These results suggest that H-2RIIBP may regulate not only MHC class I genes, but also other genes with sequences similar to region II. The role of another gene thought to regulate MHC expression, the *c-fos* gene, is also being studied, using antisense RNA as specific inhibitors.

The Section on Immunoregulation and Cellular Control, led by **Edgar Hanna**, studies the mechanisms by which microorganisms modify the immune system. The assumption being tested is that microorganisms modulate developmental and regulatory processes of regulatory T-cells. Macrophages and natural cytotoxic (NK) cells may also be affected through their regulatory cell precursors. Various bacterial toxins were used as probes to delineate this mechanism. Perpetual cell lines possessing the functions of many of the native regulatory immunocytes were cloned. These phenocopies of regulatory cells are used as targets for the bacterial toxins and subsequently tested for altered function. These clones may be stored and used as homogenous cells at representative stages of development. It was observed that the hybridoma cell line NBP2C2, which is a suppressor precursor T-cell clone, when grown in the presence of streptococcal pyrogenic exotoxin (SPE), gives rise to subclones that are no longer able to suppress antibody responses (suppressor<sup>-</sup>). Similar selection attempts in the

presence of Gram-negative endotoxin (Et) resulted in subclones with the parental phenotype. The suppressor<sup>-</sup> subclones are defective in interleukin-2 receptors and CD8 epitopes. However, the selected clones do not differ from their parent in expression of MHC haplotype and expression of an epitope of the TCR- $\alpha$  gene. Cell-free supernatants of Et treated NFR/N or nude mouse splenocytes support a transient 80-90% recovery of suppressive activity of the suppressor<sup>-</sup> subclone. Macrophage-depletion negates the activity of these supernatants. These findings largely explain the long known contra-suppressive activity of SPE and similar bacterial toxins, which appears to be at the precursor T-cell level.

## **LABORATORY OF DEVELOPMENTAL AND MOLECULAR IMMUNITY**

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## DEVELOPMENTAL ENDOCRINOLOGY BRANCH

Bruce C. Nisula, M.D., Acting Chief



Left to right: G. Cutler, G. Chrousos, F. Cassorla, B. Nisula

The Developmental Endocrinology Branch conducts basic and clinical investigations of endocrine diseases. Major emphasis within the research program is placed on adult, pediatric, and reproductive endocrinology. Within each area, the Branch employs a broad array of fundamental biochemical, pharmacological, and physiological approaches with the aim of promoting knowledge of endocrine diseases and developing new diagnostic and therapeutic techniques. Thus, a major objective in the Branch's mission in endocrinology is the translation of basic biomedical research findings into practical bedside applications through clinical research. The spectrum of endocrine disorders under study consists of those affecting the testis, ovary, adrenal, thyroid, pituitary, and hypothalamus. Of particular interest are factors regulating and diseases affecting the pituitary-adrenal, pituitary-gonadal, and pituitary-thyroid axes.

The Section on Developmental Endocrinology, led by **Gordon Cutler**, studies the basic mechanisms and clinical disorders of growth, puberty, and adrenal function. The objective of these studies is to gain new insights into basic molecular mechanisms, physiology, and pathophysiology, which can be applied to the prevention, diagnosis, and treatment of endocrine diseases, and the methods employed range from the basic approaches of molecular endocrinology to controlled clinical trials. A major strategy of the Section is to use the techniques of molecular endocrinology to gain insight into the identity and the relative importance of the growth factors and other substances that regulate epiphyseal growth.

Three major clinical trials are in progress to evaluate new approaches to the treatment of short stature. The first tests the hypothesis that prolonging the growth period by delaying puberty can enhance adult height. The observation that patients with pubertal delay due to isolated hypogonadotropic hypogonadism are significantly taller than normal subjects

provides support for this hypothesis. The second clinical trial tests the hypothesis that supplemental growth hormone (GH), with or without low dose ethinyl estradiol, will increase the adult height of girls with Turner syndrome. Since girls with Turner syndrome average more than 3 SD below the normal mean height (4' 9"), short stature is the major concern of patients with this disorder. The outcome variables include data on both safety and efficacy, and will provide a scientific basis for rational decision regarding the risk to benefit ratio of this new treatment option. The third clinical trial tests the hypothesis that supplemental GH will increase the adult height of children with non-growth hormone-deficient short stature (constitutional, familial, or idiopathic short stature). This trial will meet an important societal need for objective outcome data upon which to make rational risk-benefit analyses concerning this new treatment approach. The number of short children in the United States who are potential candidates for GH treatment is 1,000,000. Thus, the answers sought by this trial have important clinical and regulatory as well as scientific significance.

A major unresolved issue in pediatric endocrinology has been how best to establish, in a scientifically valid manner, the diagnosis of GH deficiency. A recent study from this section, published in the *New England Journal of Medicine*, showed that the mean 24-hour GH level has much lower sensitivity for the diagnosis of GH deficiency than had been previously reported. This has rekindled interest in GH stimulation tests as the most efficient and reliable means of diagnosing GH deficiency.

The molecular mechanisms involved in puberty are an area of active interest within the Section. During the current year, members of the Section cloned the human LH releasing hormone (LHRH) gene and determined the structure of its regulatory sequences. Estrogen regulates the gene both positively (5' flanking sequences only) and negatively (entire gene). The basis for the divergent effects of estrogen in different portions of the gene is presently under investigation. The positive regulation of the gene by estrogen has been localized to a cognate estrogen response element at about 450 base pairs upstream from the major transcription start site. Future studies will examine the regulatory effects of other steroid hormones and of thyroid hormone.

The differential diagnosis of delayed puberty continues to pose a difficult clinical problem for the endocrinologist. Currently, no definitive means exists to distinguish the child who has constitutional delay of puberty, and therefore will enter puberty spontaneously, from the child who has isolated hypogonadotropic hypogonadism and will require hormonal treatment to induce puberty. Recent studies in the prepubertal rhesus monkey have shown that pulsatile administration of N-methyl-D-aspartate (NMDA) can trigger pulsatile release of LHRH and puberty. This suggested that it may be feasible to develop a functional test of the integrity of the LHRH neuron based upon stimulation of the NMDA receptor. To test this concept, the naturally occurring ligands of the NMDA receptor, aspartate and glutamate, were administered to prepubertal rhesus monkeys whose gonadotroph cells had been rendered responsive to LHRH through a brief period of pulsatile LHRH pretreatment. Both aspartate and glutamate induce a brisk response of both luteinizing hormone (LH) and follicle-stimulating hormone (FSH) at an intravenous dose of 150 mg/kg. Future studies will examine the feasibility of a clinical test of LHRH neuron function based upon these observations. The hypothesis is that such a test would reveal integrity of the LHRH neuron in children with constitutional delay of puberty, and show deficient LHRH neuron responsiveness in isolated hypogonadotropic hypogonadism.

Studies of early puberty have led to recent advances in both pathophysiology and treatment. The insight into pathophysiology is the demonstration of LH-like bioactivity in the sera of boys with gonadotropin-independent familial male precocious puberty (FMPP). Current research is focussing on the chemical structure of this LH-like bioactivity. A new approach to the treatment of FMPP has proven effective in normalizing the rate of growth and bone maturation and in preventing acne, spontaneous erections, and aggressive behavior. The treatment combines an antiandrogen (spironolactone) with an inhibitor of androgen-to-estrogen conversion (testolactone) to block estrogen effects on epiphyseal growth. Current therapeutic research is directed at evaluating the long-term effects of this new treatment, at

improving treatment with more specific and/or potent antiandrogens and aromatase inhibitors, and at treating secondary central precocious puberty, when necessary, by administering an LHRH analog as well.

Ongoing studies of adrenal physiology and pathophysiology have the objective of improving the treatment of congenital adrenal hyperplasia and of improving the diagnosis, differential diagnosis, and treatment of Cushing's syndrome. One clinical trial involving congenital adrenal hyperplasia uses a combination of antiandrogen and aromatase inhibitor, which is analogous to the strategy that was employed successfully in FMPP. It is anticipated that blockade of androgen action and of estrogen synthesis will normalize growth and bone maturation, and thus permit hydrocortisone dosage to be reduced to strictly physiologic levels.

The overnight dexamethasone suppression test, which causes suppression of cortisol secretion in normal subjects but not in patients suffering from Cushing's syndrome, is employed widely as a screening test for Cushing's syndrome and as a diagnostic test for depression. Until now, it was not known whether the feedback effects of dexamethasone are exerted primarily at the hypothalamus to decrease corticotropin releasing hormone (CRH) release, or at the pituitary, to inhibit ACTH synthesis and release. To dissect these potential sites of negative feedback, basal and insulin-induced ACTH and cortisol levels were examined on a control day, after the overnight dexamethasone suppression test, and after overnight dexamethasone followed by pulsatile hCRH administration. At the hypothalamic level, overnight dexamethasone administration inhibits basal pituitary-adrenal function but not the response to insulin-induced hypoglycemia. At the level of the corticotroph, overnight dexamethasone inhibits the response to hypoglycemia, but this inhibition is prevented by pulsatile hCRH. Thus, pituitary inhibition by dexamethasone appears to result primarily from the loss of hypothalamic CRH priming rather than from a direct effect of dexamethasone on the pituitary.

Despite recent advances in the differential diagnosis of Cushing's syndrome, the distinction between Cushing's disease (ACTH-secreting pituitary microadenoma) and ectopic ACTH-secreting neoplasms remains a difficult problem. Several advances in this area have been made in the past year. First, the sensitivity of magnetic resonance (MR) scanning for pituitary microadenomas has been improved by performing the procedure with and without the contrast agent gadolinium. A single scan is not optimal because of the heterogenous nature of the pituitary lesions (e.g., solid, cystic, etc.). Some lesions are revealed by gadolinium, whereas others that are visible without contrast are obscured by gadolinium. Optimal diagnosis thus requires both types of scan. Secondly, MR scanning was found to be superior to computed tomography (CT) for the detection of ACTH-secreting bronchial carcinoid tumors within the hilar region of the lung. In this region tumors can be mistaken for blood vessels by CT, whereas MR distinguishes tumor from blood vessels, which fail to image by MR because of movement of blood in the interval before signal generation. Thus, it was found that optimal examination of the lung for ectopic ACTH-secreting tumors requires MR for the hilar region (inner 1/3) and CT for the outer 2/3, where the higher speed of CT imaging results in better resolution. In addition, increased resolution of both MR and CT scanning have resulted in improved diagnosis of micronodular adrenal disease. Although the micronodules in this condition have been regarded as too small to be imaged by radiographic procedures, it has recently proven possible to identify nodules in approximately 90% of these patients, with good correlation between the radiographically identified nodules and the nodules identified pathologically after adrenalectomy. Since the normal adrenal size in patients with micronodular adrenal disease can suggest the possibility of factitious Cushing's syndrome, the ability to diagnose this disorder radiographically will help resolve a difficult diagnostic problem.

A careful retrospective analysis of patients who have had second or third transsphenoidal explorations for Cushing's disease has provided new guidelines for when to consider this treatment approach for recurrent Cushing's disease. Although the overall remission rate was 60%, the results were considerably poorer in patients whose initial exploration revealed

tumors adjacent to the lateral walls of the cavernous sinus. By contrast, patients in whom a sphenoid sinus septum was left intact, or in whom the window in the sellar floor was small, or who had more centrally located tumors at initial exploration, had considerably better results at second exploration. Thus, based on these studies, repeat transsphenoidal surgery is no longer recommended for patients with recurrent Cushing's disease in whom the probability of tumor extension into the walls of the cavernous sinus appears high.

A major limitation in the postoperative recovery of patients with Cushing's disease, who have been cured by transsphenoidal surgery, is secondary adrenal insufficiency, which can require up to a year to resolve. To distinguish whether this adrenal insufficiency results primarily from suppression of the CRH neuron by longstanding hypercortisolism or from a direct effect of hypercortisolism on the pituitary, pulsatile human CRH was administered for one week to patients who had been cured of Cushing's disease. Pulsatile CRH administration has no effect on recovery of the hypothalamic-pituitary-adrenal axis. The response to CRH, even after one week of pulsatile administration, remains far below control levels. These observations indicate that the secondary adrenal insufficiency in these patients is associated with a profound unresponsiveness of the corticotroph cell to CRH, which cannot be corrected by one week of pulsatile CRH administration. However, this finding does not exclude the possibility of a coexisting suppression at the level of the CRH neuron. Taken together, the above work during the previous year has contributed significantly to improving the accuracy of diagnosis and the effectiveness of treatment of Cushing's syndrome, a potentially fatal endocrine disorder that often strikes children or young adults.

The Medical Endocrinology Section, directed by **Bruce Nisula**, conducts research on the glycoprotein hormones, chorionic gonadotropin (hCG), thyroid-stimulating hormone (TSH), follicle-stimulating hormone (FSH), and luteinizing hormone (LH). During the past year, this Section has performed research on a number of topics, including pregnancy loss in healthy women, developmental characteristics of the thyroid axes in childhood, structural determinants of the pharmacokinetic properties of glycoprotein hormones, and the functional significance of carbohydrate modifications with respect to glycoprotein hormone action *in vivo*.

A large population of healthy couples was studied to define the fundamental fertility characteristics of the normal population. Prior to these studies, it was known that healthy couples having intercourse regularly without contraception have about a 25% chance of achieving a pregnancy in a menstrual cycle. Of these recognized pregnancies, a percentage are lost as spontaneous abortions. A spontaneous abortion is a loss of pregnancy that has been clinically recognized by the woman and her physician. The goal of the study was to define, in addition, how often women lose pregnancies that are unrecognized by them. Such early pregnancy losses would not usually be apparent to the women because they did not miss their menstrual period or the pregnancies occurred and were lost before the expected menses. Samples and data were collected over a total of 707 menstrual cycles from 221 healthy women attempting pregnancy. The criterion for identifying a pregnancy is an hCG level exceeding 0.025 ng/ml for three consecutive days; a group of 28 women who had undergone sterilization by tubal ligation were consistently below this level. Of 198 pregnancies, 80% had detectable elevations in hCG levels by the 28th day and 50% by the 25th day of the menstrual cycle. Thus, measurement of hCG in urine allows detection of pregnancy very close to the time of implantation and prior to recognition of the pregnancy by the women (i.e., before the delay of menses occurs). The total rate of pregnancy loss after implantation was found to be 31%. Early pregnancy losses (22% of all pregnancies) are far more common than spontaneous abortions (9% of all pregnancies). Thus, healthy women conceive, progress to the implantation stage, and lose many more pregnancies than are clinically apparent. The data on these women were analyzed to evaluate the implication of a pregnancy loss for future fertility. It is clinical practice to advise women that pregnancy loss contributes to infertility. In fact, the opposite appears to be the case. Of the women who had an early pregnancy loss, 35% became pregnant in the very next cycle, 68% within three cycles, and 83% within six cycles. By comparison, in the general population 25% become pregnant in one cycle and 74% within six menstrual cycles. These data show not only that early pregnancy loss is a normal

phenomenon in healthy women attempting pregnancy, but also that early pregnancy loss tends to imply enhanced future fertility, rather than infertility. Such findings provide an important impetus for modifying current clinical practice, which has been to advise women to use birth-control methods for a while after a pregnancy loss. These findings indicate that postponement of pregnancy is an unnecessary practice, and in the older woman, it may even be detrimental.

Growth and development in children is critically dependent on normal thyroid function. Diagnosis and management of thyroid diseases in children have, until now, been hampered by a lack of crucial information on the normal development of pituitary-thyroid axis function. Information in the literature was either non-existent, incomplete, or controversial. Accordingly, the pituitary-thyroid axis function of 96 normal children, aged 5-18 years, was investigated. The circadian variation of serum TSH was examined in relation to chronological age, and its relationships to serum levels of T4, T3, and thyroxine-binding globulin (TBG) were determined. Children at all ages exhibit a circadian pattern of serum TSH concentration, characterized by a nadir in the late afternoon, a rise to a peak around midnight, a plateau for several hours, and then a decline in the daytime. The mean nadir TSH was found to be  $1.6 \pm 0.1$  in U/L, and the mean peak TSH  $3.7 \pm 0.2$  U/L, and the mean nocturnal TSH surge (% increase in TSH from nadir to peak) 144%. Neither TSH concentration nor the nocturnal TSH surge correlate significantly with age or serum thyroid hormone levels. In contrast, serum T4, T3, and TBG exhibits a significant negative correlation with age. These findings are consistent with the interpretation that developmental changes in serum thyroid hormones in children largely reflect changes in hormone transport by TBG, and are not regulated by changes in TSH secretion. This study establishes, for the first time, the normal developmental pattern of TSH in childhood, and shows that normal children have a circadian variation of serum TSH quite similar to that of adults. Thus, physicians caring for children with thyroid diseases and disorders affecting thyroid function now have available to them systematic, detailed TSH information on which to base their medical judgements. In addition to providing normative data on development, these data contribute important new evidence concerning the mechanism of the developmental changes in thyroid hormone levels in childhood. The findings point to alterations in hormone transport, rather than changes in TSH secretion as the underlying mechanism. Also of physiological interest in this investigation is the observation that the magnitude of the nocturnal TSH peak correlates significantly with the peak serum TSH after TRH infusion. Some workers have postulated that the nocturnal surge of TSH is the principal component of TSH secretion, which is regulated by increased secretion of TRH by the hypothalamus. These data lend credence to this possibility.

The structure/kinetic relationships of hCG and related molecules were investigated in the current year to assess how profound differences in carbohydrate and/or polypeptide structures affect parameters of plasma turnover of glycoprotein hormones. The impetus to the study derived in part from a controversy, recently raised in the literature, over whether the carbohydrate structure of a glycoprotein hormone is a determinant of the initial volume of distribution. Theoretical considerations dictate that the initial volume of distribution should correspond to the intravascular plasma space (volume). To address this question, the pharmacokinetics of native hCG, desialylated hCG (ds-hCG), deglycosylated hCG (dg-hCG), and the core fragment of hCG $\beta$  ( $\beta$ -core) were analyzed in male *Cynomolgus* monkeys. The metabolic clearance rates of dg-hCG,  $\beta$ -core, and ds-hCG were found to be, respectively, 15-, 47-, and 152-fold higher than that of hCG. However, despite these huge differences in kinetic behavior, the initial volumes of distribution are indistinguishable: hCG ( $0.035 \pm 0.002$  L/kg), ds-hCG ( $0.034 \pm 0.004$ ); ds-hCG ( $0.035 \pm 0.004$ ); and  $\beta$ -core ( $0.035 \pm 0.002$ ). The ds-hCG is devoid of its terminal sialic acid and has its terminal galactose exposed. The dg-hCG has lost about 70% of its carbohydrate residues, retaining for the most part its most proximal N-acetyl-D-glucosamine and N-acetyl-D-galactosamine. The  $\beta$ -core retains the trimannosyl core of the asparagine-linked carbohydrate and lacks the antennary sugar residues as well as the O-linked carbohydrate chains. Thus, as theory would predict, dramatic variations in polypeptide and carbohydrate composition do not affect the initial volume of distribution, but rather dictate profound changes in the rate constants of

disappearance. These latter parameters are presumed to reflect cellular uptake processes localized mainly to the liver, kidney, and reticuloendothelial system. These findings indicate that discrepancies in work published by others are artifactual and related either to methodological issues or purity of the glycoprotein preparations employed. In addition to resolving a fundamental question of glycoprotein hormone physiology, these studies lay the groundwork for the development of analogues with desirable pharmacokinetic characteristics for clinical applications.

Previous structure-function studies have indicated that intact carbohydrate structures are essential for the full expression of the biological effects of glycoprotein hormones. For example, removal of the carbohydrate residues from hCG (deglycosylation) drastically reduces the *in vitro* rodent gonadal cAMP response to the hormone analog; and removal of the terminal sialic acid residues from hCG (desialylation) accelerates plasma clearance and thereby reduces potency in rodent *in vivo* bioassays. This year, the consequences of these carbohydrate modifications on the intrinsic biological activity of hCG in a primate model, the male *Cynomolgus* monkey, were assessed. Large doses of highly purified dg-hCG, ds-hCG, and hCG were administered and the plasma testosterone response measured. Interestingly, the mean plasma testosterone responses over the first 6 hours achieved with dg-hCG, ds-hCG, and hCG are indistinguishable, despite the fact that the mean concentrations differ dramatically among the groups. Plasma ds-hCG and dg-hCG are undetectable by 15 and 180 min, respectively, while the mean plasma hCG is more than 2.1 nmol/L at 360 min. Whereas the *in vivo* testosterone response to dg-hCG is indistinguishable from that to hCG, their *in vitro* adenylate cyclase responses differ dramatically. The dg-hCG preparation has less than 4% of the maximal agonist activity of hCG and, in fact, is able to antagonize hCG-stimulated adenylate cyclase to approximately 6% of the maximal hCG-stimulated level. Thus, dg-hCG elicits a full testosterone secretory response in the primate *in vivo*, despite having minimal intrinsic activity and despite being a powerful antagonist of hCG action on adenylate cyclase. These findings illuminate an important attribute of the signal transduction system in the testis: Powerful antagonists of hCG action on adenylate cyclase, such as dg-hCG, are paradoxically capable of stimulating a full testosterone secretory response. The paradox derives from the fact that a minuscule amount of the intermediary messenger cAMP is sufficient to evoke a maximal and sustained testosterone secretory response—the bulk of the cAMP-producing capacity of the Leydig cell is superfluous as far as the secretory response is concerned. Earlier studies by others established the spare receptor phenomenon; more recent studies with analogues of glycoprotein hormones, such as dg-hCG, have called attention to the superfluous cAMP phenomenon and enhanced our understanding of signal transduction mechanisms.

The Section on Pediatric Endocrinology, headed by George Chrousos, investigates the hypothalamic-pituitary-adrenal (HPA) axis from physiological and pathophysiological perspectives. During the past year, the antigluccorticoid compound RU 486 and the recently discovered CRH were used to elucidate fundamental functional characteristics of the HPA axis under various physiological conditions, such as physical and emotional stress, and in major endocrine diseases and psychiatric disorders.

In dose-response studies with the antigluccorticoid RU 486 in nonhuman primates and humans, sustained elevations of plasma ACTH, cortisol, and arginine-vasopressin were observed. RU 486 binds strongly to plasma proteins, yielding a relatively long plasma half-life of about 20 h in humans. Less than 1% of the administered RU 486 is excreted in urine. This knowledge of the pharmacokinetics of RU 486 is important to devising therapeutic regimens. Treatment with RU 486 of several patients suffering from profound hypercortisolism due to the ectopic ACTH syndrome or adrenal carcinoma resulted in dramatic remissions from their clinical manifestations. Somewhat paradoxically, RU 486 is able to suppress the concentration of ACTH in the plasma of patients with adrenal insufficiency given test doses of CRH. While this result suggests some intrinsic glucocorticoid activity in RU 486, that activity is insufficient to sustain cardiovascular homeostasis in adrenalectomized monkeys. Thus, RU 486 approaches being a pure competitive antagonist at the glucocorticoid receptor. As such, it was used to address two

longstanding questions about the effects of glucocorticoids on the immune system, i.e., whether the immunological effects of glucocorticoids are receptor-mediated and whether glucocorticoids play a role in modulating the immune system at basal, resting physiological levels. In an "aseptic inflammation" rat model, in which glucocorticoids suppress exudate formation, leukocyte diapedesis, and prostanoid accumulation, RU 486 is able to antagonize the effect of pharmacologic doses of glucocorticoids, suggesting that anti-inflammatory effects are indeed receptor-mediated. In addition, RU 486 given alone causes enhancement of the inflammatory response, suggesting that endogenous glucocorticoids at physiological levels exert suppressive effects on the inflammatory response in the rat. RU 486 also causes enlargement of the thymus and spleen in these animals. These observations suggest that a glucocorticoid antagonist could be used as an immune enhancer in conditions that would benefit from such enhancement (cancer, immunodeficiency). This concept was explored in part in normal volunteers receiving RU 486 daily. The excretion of urinary free cortisol was increased markedly in these volunteers but there was no detectable change in various circulating lymphocyte markers. Similarly, there were no detectable changes in the ability of the lymphocytes to become activated and to become more cytotoxic. These findings suggest that in primates glucocorticoid does not affect the immune system in the basal state, a significant difference from rodents. In other studies, RU 486 was instrumental in identifying a central nervous system defect in rats prone to arthritis in response to antigenic stimulation with streptococcal cell wall polysaccharide. These rats (Lewis rats) have deficient CRH, ACTH and glucocorticoid responses to stressors. A finding of particular interest is that histocompatible rats (Fisher rats), which normally do not develop arthritis in response to streptococcal cell wall polysaccharide, do so when treated with RU 486. The work outlined above has established that RU 486 carries promise as a diagnostic and therapeutic tool and as a probe to study adrenal and gonadal physiology. As a challenger of the hypothalamic-pituitary-adrenal axis, it provides a key tool in the study of disorders of the axis, such as Cushing's syndrome, adrenal insufficiency, psychiatric hypercortisolism. As a therapeutic agent it appears to be useful in treating a subset of patients with hypercortisolism. As an experimental tool, it provides an excellent means to probe stress physiology and the role of glucocorticoids upon the immune system and the inflammatory response.

Hypothalamic CRH and the glucocorticoid receptor are the main regulators of the HPA axis. CRH has given great impetus to stress research and to the study of pediatric psychiatric disease. In addition, its availability has revised the way we evaluate and treat the major diseases of the HPA axis, including Cushing's syndrome (hypercortisolism), and adrenal insufficiency. Dr. Chrousos and his colleagues have pioneered the application of CRH to the differential diagnosis of major endocrine diseases, to elucidating pathophysiological mechanisms in major endocrine and psychiatric disorders, and have explored its role in responses to physical and emotional stress. With respect to clinical diagnostic applications, the CRH stimulation test has significantly improved our capacity to differentiate the causes of Cushing's syndrome and, hence, to treat successfully its various forms early in their course. The CRH test also proved to be an excellent means of differentiating between the primary, secondary, and tertiary forms of adrenal insufficiency. Moreover, in patients with hypercortisolemic psychiatric disease, the CRH test and measurement of CRH in the cerebrospinal fluid suggest a major pathophysiologic feature of these states—hyperfunction of the CRH neuronal system. This finding is particularly important because CRH appears to be a major biochemical coordinator of the stress response; hence, it probably not only stimulates ACTH and glucocorticoid secretion, but also activates the central sympathetic and arousal systems, and produces characteristic behavioral and biochemical changes consistent with chronic stress and the syndrome of depression. As an example, the central administration of CRH results in anorexia, decreased libido, hypothalamic hypogonadism, and anxiety, all of which are among the cardinal manifestations of major depression. In addition, CRH given intracerebroventricularly to experimental animals produces limbic seizures that cross-sensitized with electrically-kindled seizures. This finding may be relevant to the natural history of recurrent affective disorder, which is characterized by a gradual increase in the severity and frequency of episodes throughout its course. As a corollary, it has been known since the mid-sixties that infant experimental animals that are separated from their mothers for prolonged periods show an accentuation of hypothalamic-pituitary-

adrenal responsiveness to stress throughout life. Interestingly, they also show increased vulnerability to the sensitization of key limbic sites induced by CRH.

Studies of the regulation of hypothalamic CRH secretion in both *in vivo* and *in vitro* models have yielded new insights. They have demonstrated that the hypothalamic CRH neuron is under the stimulatory influence of serotonergic, cholinergic, norepinephrinergic, dopaminergic, epinephrinergic and histaminergic neurons and under the inhibitory influence of GABA/benzodiazepine and opioid peptide systems. The CRH neuron appears also to be regulated by a series of negative feedback loops: a CRH-mediated (ultrashort) loop, an ACTH- and  $\beta$ -endorphin-mediated (short) loop and a glucocorticoid-mediated (long) loop. The fact that norepinephrine stimulates CRH secretion has settled a long-standing controversy; moreover, this finding not only provides a putative mechanism for the concordance in the activation of the sympathetic nervous system and the HPA axis during stress, but also provides a more firm rationale for the mechanism of action of many antidepressant and anxiolytic medications.

Studies of the interaction between the immune and CRH systems have also yielded novel findings in the past year. Several cytokines, including interleukin I, tumor necrosis factor- $\alpha$ , as well as several lipid mediators of inflammation, cause profound stimulation of the HPA axis via activation of the CRH neuron. Thus, it appears that the CRH neuron provides a link between the immune and central nervous systems, by which cytokines/lipids stimulate CRH-mediated glucocorticoid-induced negative feedback of the inflammatory/immune response. Bearing importantly on the concept of a link between the immune system and the HPA axis are the results of studies of the animal model discussed above, in which autoimmune arthritis is associated with a poor CRH response to inflammatory mediators and subsequent hyper-responsiveness of the immune system. This suggests a novel mechanism for the pathogenesis of autoimmune disease.

The HPA axis plays an integral role in the normal physiology of pregnancy, labor, and delivery. Current studies have shown that the placenta secretes CRH as well as ACTH and  $\beta$ -endorphin. The production of these hormones was shown to increase gradually in the third trimester of pregnancy and then increase further in labor. It appears that there is a paracrine/autocrine regulatory loop in the placenta with CRH stimulating local secretion of ACTH and  $\beta$ -endorphin. Furthermore, the HPA axis may be involved in the immunosuppression known to occur in pregnancy and in the development of the post-partum depression syndromes, both of which are under study.

The Section on Steroid Hormones, led by Lynn Loriaux, has as its objective the furthering of our understanding of the role of steroid hormones in the complex processes of growth, development, and reproduction, to understand the diseases associated with these processes and, where possible, to initiate and to evaluate rational therapy for them. The problems currently being addressed include delineation of the mechanisms of glucocorticoid resistance, development of novel methods for quantitation of steroid secretion in various diseases, and elucidation of the role of progesterone in the menstrual cycle and in the regulation of specific endometrial proteins.

The glucocorticoid receptors from two models of glucocorticoid resistance have been studied. The gene encoding the glucocorticoid receptor from New World primates has been cloned and sequenced. Multiple base differences became evident when a New World species gene was compared with an Old World species gene, in particular, in the steroid binding region and in the region that is thought to bind heat shock protein, potentially an important cofactor in glucocorticoid action. Such differences were not found when the receptor from a common human resistant state, Cushing's Syndrome, was examined. Preliminary studies of size and RFLP patterns revealed no differences between the receptors from these tumors and receptors from adjacent normal tissue. Studies in the coming year will focus on identifying the mutation in the New World primate that is responsible for the glucocorticoid resistance by synthesizing glucocorticoid receptors with promising mutations and examining their action using a standard reporter gene technique.

Studies of the cortisol production rate using a deuterated trace cortisol molecule in normal subjects and patients with Cushing's Syndrome have demonstrated complete separation of the two groups and have provided a benchmark for the evaluation of the standard tests employed for the diagnosis of Cushing's Syndrome. An unexpected result was the finding that the levels of 17-hydroxy steroids correlate better with the cortisol production rate than does the urinary free cortisol concentration.

Studies in a primate model of depression have focused on elucidating the mechanism of increased cortisol production in these animals. Preliminary studies by this group have attempted to clarify whether or not the pituitary ACTH secretion characteristics of the disorder are CRH dependent. Inferior petrosal sinus sampling has revealed that CRH is easily measurable in these animals, suggesting that the hypercortisolism of affective disease is hypothalamic in origin.

Progesterone is essential for the normal morphologic development of the luteal phase endometrium, for implantation of a fertilized egg and for maintenance of pregnancy. Given its critical role in reproduction, surprisingly little is known about the mediator(s) of progesterone action. In the current year, a project headed by Lynette Nieman addressed this issue using a recently described progestin-dependent glycoprotein, placental protein 14 (PP14). These studies suggest that serum PP14 concentrations reflect previous exposure to endogenous progesterone, and that delayed endometrial maturation is associated with low PP14 and progesterone values. Further studies in infertile women and those with multiple miscarriages should give better insight into the cause and, possibly, a treatment for these disorders. Guinea pigs, like women, require progesterone for normal endometrial development, implantation and pregnancy. The antiprogesterin RU 486 was employed to probe the process of implantation in this animal model. Small daily doses of the drug result in a marked decrease in implantation sites, confirming the critical role of progesterone in nidation. Studies in non-pregnant women not at risk for pregnancy showed that small daily doses of RU 486 delay the normal maturation of the endometrium without significantly changing the hormonal patterns or timing of the menstrual cycle. No adverse effects were seen in either study. Treatment with RU 486 is a model for corpus luteum deficiency, which holds promise for helping elucidate how this condition results in infertility.

The Unit on Linear Growth Physiology, directed by **Fernando Cassorla**, investigates the hormonal mechanisms that are responsible for linear growth. Principal areas of investigation in the current year include improving the accuracy of the methods employed to diagnose GH deficiency, studying the effects of GH and sex steroid administration on linear growth in patients with delayed puberty, and determining the cortisol production rate in normal children and adolescents. In other studies, pubertal delay is being induced in children with extreme short stature with the objective of prolonging prepubertal growth prior to the pubertal spurt and possibly enhancing ultimate height by delaying epiphyseal fusion. The impact of GH therapy on the adult height of non-GH deficient children with short stature is being investigated through a randomized, double-blind, placebo-controlled clinical trial. Finally, the effect of growth hormone-releasing factor (GHRH) on linear growth in GH-deficient children is being determined under different treatment regimens in order to optimize growth.

The daily cortisol production rate in normal children and adolescents was determined using a stable isotope-dilution technique employing high-performance liquid chromatography-mass spectrometry (LC/MS), in collaboration with the LTPB. Thirteen normal subjects, ages 8-16 years, were studied. Deuterated cortisol was infused continuously for 30 hours at 5% of the anticipated daily cortisol production rate. After 6 hours of tracer infusion, which were needed to achieve steady-state levels, blood was obtained every 20 min for 24 hrs. Deuterated cortisol dilution in plasma was determined by LC/MS. The cortisol production rate is  $9.8 \pm 2.7$  mg/day (mean  $\pm$  SD) in the normals and does not vary with sex. The cortisol production rate was found to be  $10.1 \pm 2.9$  mg/M<sup>2</sup>/day in 7 prepubertal children, and  $10.0 \pm 3.0$  mg/day in 4 Tanner V adolescents. After correcting for surface area, the cortisol

production rate is  $8.5 \pm 2.3$  mg/M<sup>2</sup>/day in the Tanner V adolescents ( $p < 0.05$ ). These results suggest that the cortisol production rate in children and adolescents is significantly lower than previously estimated. The implication of these data is that the replacement doses of glucocorticoids currently used in the treatment of adrenal insufficiency need to be carefully reevaluated. When patients are given replacement glucocorticoids at the currently accepted dose of 12 mg/M<sup>2</sup>/day, signs of glucocorticoid excess may develop. This has profound implications for the management of children with adrenal insufficiency, whether associated with panhypopituitarism or congenital adrenal hyperplasia. It is expected that prescribing more physiologic replacement doses of glucocorticoids will allow these patient to grow better and to avoid developing evidence of glucocorticoid excess.

Boys with isolated hypogonadotropic hypogonadism and those with anencephaly may show microphallus and subnormal testicular growth at birth. The failure to virilize fully has been attributed to the lack of gonadotropic or testosterone stimulation in the latter part of gestation. In the current year, a primate model of this human condition was devised by suppressing pituitary-gonadal activity with a long-acting LHRH analog (LHRH<sub>A</sub>) from mid-gestation through early infancy in male *Cynomolgus* monkeys. The effects of this suppression on sexual development and growth were carefully monitored. Thus, D-Trp<sup>6</sup>-Pro<sup>9</sup>-NET-LHRH in microspheres were injected subcutaneously into fetal *Cynomolgus* monkeys, and the external genitalia, weight, linear growth, and pituitary-gonadal function of the 2 groups were compared with a placebo-treated group. As expected, plasma FSH, LH and testosterone levels were found to be lower in the LHRH<sub>A</sub>-treated group than in the placebo group. The striking finding was that the testicular and phallic size in the LHRH<sub>A</sub>-treated group were significantly less than in a placebo group. The results clearly show that suppression of the hypothalamic-pituitary-gonadal axis *in utero* leads to impaired phallic and testicular growth during early infancy in *Cynomolgus* monkeys. This model shows that gonadal stimulation by the fetal pituitary during the latter half of gestation is responsible for penile and testicular growth. Dr. Cassorla and his colleagues are now determining whether this pituitary-gonadal activation *in utero* has any long-term fertility effects by studying the pubertal development and reproductive function of these animals. This work will help us to understand the significance of pituitary-gonadal activation *in utero* and the consequences of its derangement, such as those observed in patients with hypogonadotropic hypogonadism.

The Unit on Reproductive Endocrinology, directed by George Merriam, investigates the regulation of reproductive function and the neuroendocrine control of gonadotropins and of growth hormone. Studies in reproduction emphasize the neuroendocrine control of the normal menstrual cycle, and the disorders of hypothalamic amenorrhea, hypogonadism, luteal phase deficiency, premature ovarian failure, and ovarian hyperandrogenism.

Previous studies by this group have shown that, in the normal menstrual cycle, a small rise in progesterone precedes the ovulatory midcycle LH surge, and may be the ovarian signal that the dominant follicle is ready to ovulate. To determine if this is so, normally cycling women received 1 mg of the progesterone antagonist, RU486, or placebo daily in the late follicular phase of the cycle. Although ovarian follicular development continued unimpeded, ovulation was delayed by 3-4 days in the treated group. When the experiment was repeated in 5 women with hypothalamic amenorrhea treated with pulsatile GnRH, a similar delay was observed, indicating that hypothalamic effects are unnecessary to the action of progesterone, which therefore probably occurs at the pituitary level. When supplemental progesterone was given along with RU486, the delay in ovulation was reversed, confirming the specificity of the effect. Thus, progesterone secretion may well serve as an important regulatory signal.

Seasonal changes in reproductive function dominate the regulation of breeding in many species, but the role of photoperiod in the control of reproduction in man and higher primates is unclear. A series of studies in rhesus monkeys, housed under controlled environmental conditions and artificial lighting, has demonstrated for the first time that photoperiod is also a potent regulator of reproduction in primates. When the lighting pattern was switched from short to long days (16 hours of light per day), prolactin levels and gonadal function were suppressed; the switch from long to short days (8 hours of light) reversed this

and reactivated gonadal function. When the photoperiod was held constant, without periodic adjustments, rhythmic changes in reproductive status still occurred, suggesting the presence of an endogenous circannual rhythm. This is the longest endogenous periodicity to be demonstrated in higher animals.

The suppression of cyclic menses during lactation is one of the major factors affecting the spacing of children in most of the world; yet the mechanism of this effect is poorly understood. Effects at CNS, pituitary, and gonadal levels have all been postulated. Initially, levels of prolactin are elevated, and a parallel has been drawn to the amenorrhea of prolactinoma patients. Subsequently, prolactin falls, but amenorrhea persists as long as suckling continues. Studies under way are aimed at elucidating the mechanism of postpartum amenorrhea. These subjects show a marked suppression of pulsatile gonadotropin secretion during lactational amenorrhea, with recovery as soon as suckling ceases. Treatment with pulsatile GnRH during lactation results in normal follicular development, ovulation, and a normal luteal phase, essentially ruling out an ovarian abnormality. Thus lactational amenorrhea appears to be an entirely neuroendocrine phenomenon. Companion studies in animals are examining the changes in mRNA for gonadotropin-releasing hormone during lactational hypogonadism, using *in situ* hybridization histochemistry to determine if the altered function is regulated at this level.

Luteal phase dysfunction is a poorly understood syndrome. It has been proposed that inefficient endometrial maturation accounts for a failure of normal implantation. This could be due to insufficient progesterone secretion by the corpus luteum, or an inadequate endometrial response. The diagnosis of luteal phase dysfunction is uncertain, and is currently based upon endometrial biopsies, which are painful and not well calibrated. Previous work by this group has shown that progesterone secretion in the luteal phase is pulsatile; thus, single measurements of progesterone can be very misleading, and measuring integrated progesterone secretion is not practical for clinical purposes. Since luteal function remains under LH control, in principle the response to a standardized gonadotropin challenge could provide a better calibrated measure of corpus luteum status. Studies now under way show that administration of hCG can provide a reliable stimulus to progesterone secretion, with less variability than that obtained from single measurements, and that responses are low in patients with abnormal endometrial biopsies. If confirmed, these studies could lead to a non-invasive test of luteal function for use in the evaluation of infertile women.

Menopause normally occurs after age 50, and a cessation of menses before age 40 is termed premature ovarian failure. Generally, this condition is considered to represent an irreversible depletion of ovarian follicles, like normal menopause. However, this is not always so, and remissions of ovarian failure with resumption of ovulation have been reported, occurring either spontaneously or after estrogen replacement. Autoimmune ovarian disease has been reported as a cause of premature ovarian failure, and it is likely that many patients with idiopathic ovarian failure also have autoimmune oophoritis. The association of remission with estrogen therapy suggests that not all follicles are destroyed, but only those stimulated to grow by gonadotropins. This makes it credible to hypothesize that gonadotropin stimulation leads to expression of the antigens which are targets of the autoimmune process. By removing gonadotropin stimulation, one might reduce the antigenic burden and allow a temporary remission to occur. Several studies are underway to test this hypothesis in patients and animals, and to study the mechanisms involved.

Hyperandrogenism is a common cause of oligomenorrhea and infertility, but still poses etiologic, diagnostic, and therapeutic challenges. An association of polycystic ovary syndrome with insulin resistance or obesity suggests a link with hyperinsulinism, perhaps by action at the IGF-1 receptor, but this is unproven. A whole-follicle ovarian organ culture technique has been developed to allow studies of the effects of changes in IGF-1 and other growth factors on thecal transformation of ovarian stromal fibroblasts. A study of ovarian function and morphology in patients with acromegaly and high IGF-1 levels showed a high incidence of polycystic ovaries, consistent with the hypothesis that IGF-1 is indeed involved in the control of androgen production.

Another major area of interest of this group is the neuroendocrine control of GH, and the diagnostic and therapeutic applications of GHRH. Not only is pituitary GH essential for normal growth, it is also important in the normal regulation of metabolism. GHRH and somatostatin (SRIF) are the two hypothalamic peptides that together regulate GH synthesis and secretion. A principal aim is to explore the role of GHRH and related peptides in the treatment of GH deficiency and GH excess. Early studies from this group showed that intensive treatment with multiple daily doses of GHRH can stimulate GH secretion and restore growth in most children with GH deficiency. This demonstrates that most cases of GH deficiency represent a hypothalamic deficiency of GHRH, and that the pituitary can be stimulated to produce sufficient quantities of GH to support normal growth. These intensive regimens are sufficiently complex to be impractical by comparison with conventional GH treatment regimens. Current studies have now demonstrated that even a single daily dose of GHRH can restore normal growth in these patients, a regimen that is as simple but more physiologic than conventional treatment with GH. It also appears possible that co-administration of a drug that suppresses SRIF (SRIF blocks GH secretion) may enhance the therapeutic response to GHRH and further improve growth.

GHRH, the physiologic stimulator of GH release, is a 44-amino acid peptide. A hexapeptide derived from enkephalin and unrelated to GHRH has been shown to have potent GH-releasing activity in primates and man, and can stimulate a much greater rise in GH than can GHRH itself. Its dose-response curve however, is not parallel to that of GHRH, indicating a different mechanism of action. Both the mechanism of action and potential clinical utility of this peptide are under study. Its action at the pituitary level differs from that of GHRH, with much less stimulation of cyclic AMP. It appears that in addition to pituitary effects, the hexapeptide stimulates the release of GHRH from the hypothalamus *in vitro*, and its effects in animals are blocked by inhibitors of GHRH secretion. This dual effect may account for its high potency *in vivo* and its differences from GHRH.

Overproduction of GH is most commonly due to pituitary tumors and can cause crippling deformities. Conventional therapy of these tumors with surgery, radiation, or drugs is frequently unsuccessful. Work in this group has shown that these tumors remain sufficiently well differentiated to respond to GHRH with a rise in cyclic AMP and GH. When the tumors cannot be visualized in patients using conventional imaging techniques, measuring GH in blood sampled from both inferior petrosal sinuses can indicate on which side the tumor is located, facilitating tumor removal. Progress has also been made on a novel approach to therapy of these tumors. GHRH is linked to a toxin which would then be concentrated in tumor cells, allowing them to be killed. An organoboron conjugate of GHRH as been synthesized, which emits lethal, but very localized,  $\alpha$  radiation upon exposure to neutrons, and has been found to be biologically active and concentrated in pituitary cells. Its ability to induce cytotoxicity in pituitary cells is being tested.

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## LABORATORY OF COMPARATIVE ETHOLOGY

Stephen J. Suomi, Ph.D., Chief



Left to right: M. Bornstein, D. Symmes, J. Newman, F. Pedersen, S. Suomi, M. Lamb

The Laboratory of Comparative Ethology (LCE) carries out a program of research investigating behavioral and biological development in humans and in nonhuman primates. The influences of both genetic and environmental factors, and their multiple interactions, are explored in a comparative approach in order to characterize the origins, ontogeny, and evolution of various behavioral phenotypes. Experimental results in nonhuman primates are correlated with findings from longitudinal studies of human infants and their families, as well as with data obtained by neuroscience techniques. Longitudinal designs are employed to address issues of ontogenic continuity *vs* change, and in many of the investigations a variety of both behavioral and biological measures are collected concomitantly. A major emphasis is placed on characterizing and understanding normative patterns of biobehavioral development, so that deviant patterns can be readily identified and their consequences evaluated with respect to established norms.

The Comparative Behavioral Genetics Section (CBGS), headed by **Stephen Suomi**, investigates various processes underlying biological and behavioral development in selected nonhuman primate species by focusing on interactions between genetic and environmental factors that affect the course of an individual's ontogeny. Research the past year continued its primary focus on characterizing individual differences among rhesus monkeys in their biobehavioral responses to environmental novelty and/or challenge, determining how genetic and environmental factors interact to shape such individual differences, and assessing the long-term developmental consequences of these different response styles. In one major study cross-fostering techniques were utilized to evaluate the effects of different types of maternal care on rhesus monkey infants selectively bred to be unusually reactive to novelty or challenge ("uptight" phenotype), compared with infants selected to display more normal biobehavioral response styles ("laid-back" phenotype). Thus experienced multiparous monkey females who

differed systematically in their characteristic maternal "style" raised infants who differed in their genetic risk for extreme reactions to environmental novelty and challenge. Infants whose pedigree put them at either high or low risk for reactivity were cross-fostered within the first 4 days of life to these multiparous females, who either had consistently demonstrated a highly nurturant style of care toward previous offspring or who had demonstrated species-normative levels of support, punishment, and rejection.

Analyses completed this year revealed that high-reactive infants differ systematically from control infants in their neonatal test profiles but display normal patterns of behavioral development during their 6 months with foster mothers. In fact, high-reactive infants cross-fostered with nurturant females actually exhibit accelerated developmental curves relative not only to high-reactive infants reared by control foster mothers but also to control infants reared by either kind of foster mother. However, when challenged by brief maternal separation at 6 months of age, high-reactive-pedigree infants display extreme behavioral and adrenocortical reactions regardless of their respective foster mothers. By contrast, control infants exhibit much milder reactions to challenge, but only if they have been reared by nurturant foster mothers. These behavior patterns are reversed immediately after reunion. Long-term follow-up of the first cohort of these cross-fostered monkeys, now entering puberty, has revealed that the unexpected social advantage apparently conveyed to high-reactive youngsters by nurturant foster mothers clearly carries over to subsequent situations in which social support networks are available, but not to other situations. These findings provide a clear and important demonstration that genetic predispositions can be modified substantially by specific environmental events during development and that the modification depends both on previous experiences and on the current environmental situation.

Data from a second ongoing longitudinal study of individual differences in biobehavioral responses of rhesus monkeys to challenge has provided compelling evidence that such individual differences also occur among wild-living monkeys and are likely to be of substantial biological significance. In this study young rhesus monkey males living in a free-ranging natural troop on Cayo Santiago, PR were studied as they passed through puberty, a period during which males in the wild typically emigrate from their natal troop and attempt to join new social groups. Measures of cardiovascular, adrenocortical, and behavioral response to environmental challenge were obtained from these pubertal males during each of two brief periods of capture and physical restraint for routine annual veterinary examinations. In addition, daily behavioral observations were carried out on each subject before, during, and following emigration from its natal troop. Thus, it was possible to characterize each of these young males in terms of relative biobehavioral reactivity to a standardized challenge and then monitor their activities in a wild setting during a crucial period of developmental transition.

Results to date have revealed substantial individual differences among these wild males in their biobehavioral response to the capture-and-restraint challenge, with values that mirror those seen in captive pubescent rhesus monkey males—and with remarkable year-to-year stability of these individual differences. Strikingly, high-reactive males (i.e., those with higher and more stable heartrates, lower vagal tones, and higher levels of plasma cortisol) tend to leave their natal troop later chronologically than low-reactive males. Moreover, when they finally do leave their natal troop, these high reactive males usually fail to join all-male "gangs," as is typical for most adolescent males emigrating in feral settings, but instead try to go directly into a new troop, albeit limiting their interactions largely to low-ranking members on the periphery of the new troop. Ongoing observations will permit determination of the relative success of the different emigration "strategies" displayed by these high and low reactive males. The discovery of different male dispersal strategies associated with differential biobehavioral reactivity to brief challenge is especially striking in that other more "traditional" measures of these males' chronological, physical, and hormonal maturity, as well as social status in their natal troop, failed to predict these different strategies. Thus, behavioral and biological assays developed to characterize relative reactivity among laboratory-reared captive rhesus monkeys not only can be used to demonstrate similar patterns of interindividual variation in wild populations, but they also

turn out to be the best current predictors of individual patterns of dispersal among pubertal males growing up in feral troops, a topic of intense current interest among behavioral ecologists and evolutionary biologists.

Given these and other findings documenting major sex differences in rhesus monkeys clearly emerging around puberty, the CBGS this year directed increased resources toward characterizing species-normative patterns of biobehavioral continuity and change throughout the transition through puberty. Data collected in long-term longitudinal studies documented systematic changes in several different biobehavioral systems that appear during adolescence. First, results from 3 independent longitudinal samples of subjects growing up in different settings demonstrated an apparent suppression of adrenocortical response to environmental challenge in male, but not female, monkeys as they enter adolescence. Pubertal males born and raised in a wild rhesus monkey troop, as well as laboratory born males either reared by their biological mothers in age-graded social groups or hand-reared in a nursery and subsequently put into mixed-sex peer groups, all show significant decreases from juvenile levels of plasma cortisol following challenge, whereas most females either exhibit stable or developmentally increasing cortisol values. In a similar vein, as rhesus monkey males approach adolescence, they have significantly greater drops in CSF levels of the serotonin metabolite, 5-HIAA, than do females, a pattern that closely parallels developmental increases and emerging sex differences in the absolute frequency and relative intensity of aggressive behavior.

These characterizations of normative patterns of biobehavioral functioning during a period of major developmental transition have provided two new insights. First, the most dramatic developmental changes in measures of behavior, adrenocortical and monoamine pathway function observed in rhesus monkeys seem to precede the most obvious changes in physical features and sex steroid levels, in some cases by as much as a year. This is not true for other biobehavioral measures generally sensitive to environmental challenge, such as levels of plasma ACTH (adrenocorticotropin) or CSF MHPG (a norepinephrine metabolite). Second, there is remarkable stability of individual differences in each of these disparate measures of response to challenge prior to and generally throughout the pubertal period, despite the dramatic changes in absolute levels of these measures during that same period. Such findings suggest that individuals who will exhibit extreme reactions to challenge during the relatively turbulent times of adolescence can be identified well before the onset of obvious physical pubertal change.

A quite different set of studies investigating patterns of response in the face of environmental novelty focused on biobehavioral consequences of various efforts to "enrich" the living environments of captive primates, a research topic particularly relevant in light of recent amendments of the Animal Welfare Act concerning the maintenance of nonhuman primates in captivity. Four different forms of "environmental enrichment" were systematically introduced to monkeys living in otherwise stable physical and social environments. In each case, these fundamentally different enrichments—a fleece-covered "grooming board" for individually housed adults, a 1/4-acre enclosure periodically available to members of family groups, a provision of cover designed to promote foraging behavior among social group members, and systematic distribution of preferred food items within "foraging bins"—clearly altered behavioral profiles of most subjects in directions consistent with current notions regarding well-being in captive nonhuman primates. These findings have already led to specific changes in some Standard Operating Procedures for animal care in the LCE primate colony.

Finally, several investigations continued to characterize individual differences in reflex development, activity state patterns, and temperament during the neonatal period among nursery reared rhesus monkey infants and selected comparison species. A major goal of these research efforts centers around the use of neonatal measures to predict various patterns of individual differences during later developmental periods. In one study, differences among rhesus monkey infants in fussiness during the first month were associated with the relative power of environmental enrichment to enhance cognitive and social-emotional

development—fussy (high-reactive) infants benefited less from two types of extra environmental stimulation than did control rhesus monkey infants. A second study, in collaboration with the New England Regional Primate Center, discovered that different nursery procedures (for example, amount of handling during feeding) are associated with differences in neonatal test scores, in some cases exceeding differences between infants of different macaque species reared in the same nursery environment. The significance of these latter findings lies in the demonstration that neonatal temperamental characteristics are apparently subject to environmental modification within the first few days and weeks of life, at least in macaque monkeys. Other collaborative studies utilized standardized neonatal assessments to evaluate the efficacy of prenatal intervention for hydrocephalus in rhesus monkey infants, to predict cognitive and social-emotional functioning in young chimpanzees, and to establish normative developmental standards for captive *Cebus apella* infants.

The Section on Social and Emotional Development (SSED), headed by **Michael Lamb**, studies the effects of different types of caregiving practices on infant and toddler social and emotional development and cognitive competence. Special attention is given to longitudinal approaches that involve cross-cultural comparisons and those examining nonnormative samples of both parents and infants. A major topic of research during 1989 was to characterize individual differences in early reflex development, patterns of arousal and activity, and temperament in early infancy, but in this case among selected samples of young human children. Just as in the previously described rhesus monkey studies, the overall objectives of these very early assessments of human infants have been both to understand the interrelationships among different behavioral and physiological systems at this stage of development and to determine the relative efficacy of these various measures in predicting patterns of individual differences later in infancy and into childhood.

In one major study, 5-month-old infants selected on the basis of parental reports of extreme behavioral and physiological arousal during their initial months (e.g., frequent constipation or colic, sleeping difficulties, respiratory or skin allergies, strongly adverse behavioral reactions to environmental changes, and extreme irritability) were compared with same-age control infants in terms of a variety of psychophysiological measures (heart rate, heart rate variability, and vagal tone), levels of saliva cortisol obtained in a controlled laboratory setting, temperament (as assessed by a standardized maternal questionnaire), and daily activity patterns (as recorded by mothers in diary fashion over a 1-week period in the home). The infants were then followed longitudinally in standardized laboratory sessions and home visits at 7, 10, and 13 months of age in order to obtain additional age-appropriate measures of cardiovascular and adrenocortical activity, temperament and emotional responsiveness, and interaction patterns and attachment relationships with their mothers. Preliminary analyses of data from the initial phase of the study revealed that infants who were described as extremely irritable by 4 months of age score higher than nonirritable babies on the fussy and unadaptable subscores of the temperament index when they are 5 months old. Irritable babies are more likely to have extremely high or low vagal tone, and extremely high or low heart rate, than are nonirritable babies. Additionally, infants with extremely high or low vagal tone or heart rate appear more fussy and unadaptable on the relevant temperament subscales. Finally, children characterized by symptoms of irritability differ significantly from children with no such symptoms on a derived measure representing the absolute distance from the grand mean of the vagal tone measure. Only scores on this derived variable accounted for a significant amount of variance between the two groups (irritable vs not irritable) in a step-wise discriminant function analysis. These findings are significant in that they demonstrate convergence of both behavioral and physiological indices of extreme patterns of fussiness and arousal by 5 months of age, considerably earlier chronologically than has been reported for human infants in previous studies. Comparisons of these 5-month data with the results of the subsequent laboratory and home visits are currently underway.

A second area of interest common to SSED researchers and previously described nonhuman primate work is developmental continuity and change through puberty and adolescence and into early adulthood. The goals of these studies include not only identifying normative patterns of social, emotional, cognitive, and physiological functioning in adolescence but also

identifying aberrant or extreme patterns and determining the long-term consequences. One aspect of this work involves use of large scale national surveys of adolescent youth and their children, especially as they relate to teenage pregnancy and parenthood. Analyses of survey data completed during the past year indicate that teenage mothers are much more likely to exhibit a greater array of antisocial, educational, and substance abuse problems prior to pregnancy than are adolescent females who do not become pregnant but are otherwise matched for age, race, and socioeconomic background. This pattern of early problems is very similar to that previously reported for adolescent males who become fathers during their teens. Moreover, in appropriately matched comparisons with adolescent females who subsequently either have their first child between 19 and 21 years of age or who are still childless at 21, teenage mothers are more likely than either young adult mothers or non-mothers to have records of school suspension, truancy, running away, smoking marijuana, and fighting. There are variations in the results by race and geographical area: Blacks report fewer problem behaviors than do Whites, and urban women engage in fewer problem behaviors than rural women. Young urban women who engage in three or more problem behaviors are more likely than women who report no problem behavior to subsequently have a child prior to age 19. These findings contradict the notion that adolescent parenthood is a random event, with some proportion of sexually-active youngsters unlucky enough to get caught. In fact, the findings suggest that adolescent parenthood is but one symptom of psychosocially-troubled youths, illustrating the multi-problem nature of adolescent parenthood and the need for a clear and comprehensive understanding of the context of adolescent pregnancy.

In a related study, national survey data were utilized to evaluate long-term consequences of adolescent parenthood and marriage. A striking finding is that adolescent marriage is associated in both men and women with deficits in marital stability, income, educational attainment, and occupational prestige up to 40 years after the marriage. Clearly, adolescent marriage can have adverse consequences that endure throughout men's and women's working lives. This finding is especially noteworthy because it has often been assumed that men are spared adverse effects because they do not have to endure the pregnancy and traditionally play a minor role in childcare.

A third major research focus of the SSED during 1989 was a continued longitudinal study of the effects of different types of infant care arrangements (home care, family daycare, or center-based daycare) on social, personality, and intellectual development in a large, carefully selected sample of toddlers. The different care arrangements began when the children were 16 months old, and annual assessments of their social, emotional, and cognitive patterns and capabilities have continued to the present (these children are now beginning their first grade of school). Results of data analyzed to date have consistently demonstrated that the type of care arrangement by itself is not associated with major differences in child outcome measures at any time during the toddler and young childhood years. In contrast, by the beginning of the second year of assessment, quality of home care (itself correlated with family social class), infant temperamental difficulty, and perceived social support show an effect. Two years after enrollment, quality of home care continued to have the most consistent impact on personality maturity and emergent social skills with peers and adults. The quality of alternative care now also had an effect, albeit one that is inconsistent and more modest than that associated with quality of home care. The impact of the quality of alternative care becomes more dramatic when the indices of quality are expanded to include observational measures of the children, careproviders, and settings. Later analyses examining children's compliance with maternal requests in a task-like setting revealed that the quality of home care is the best predictor of both the mothers' and children's behavior. The same pattern of relative influence was also found for measures of cognitive capabilities, with quality of home care accounting for the largest share of the variance at each age tested. The quality and extent of nonparental care has smaller but significant impacts as well. Ongoing research is designed to further explore the determinants of individual differences in the mothers' behavior—a key component of the quality of home care.

In view of these and other findings demonstrating the importance of various aspects of home care during infancy, toddlerhood, and early childhood, the SSED has initiated a series of cross-cultural studies designed to characterize various aspects of the home environment across different social and physical ecologies, especially in terms of parental beliefs and values, parenting programs, and how differences in these domains affect children's development. In the first study, SSED staff collected interview data about perceptions, values, expectations, responsibilities, and practices of West African parents. These data are being analyzed and will be interpreted in the context of information about variation in developmental niches. In a second study completed this year SSED staff explored the effects of agreement between Swedish mothers and fathers regarding socialization values. Agreement between parents is less clearly associated with developmental outcomes in Sweden than in the USA. In a third study, SSED staff are attempting to assess specific maternal and child attributions about one another in order to identify the extent to which attributions or expectations shape the way grade school-age children interact with their parents. Finally, SSED staff are exploring the validity of Strange Situation assessments of infant-mother attachment security in Japanese dyads.

The Child and Family Research Section (CFRS), headed by Marc Bornstein, examines perceptual, cognitive, and dispositional development in human infants and children, with special emphasis on studying the relationships among early attentional processes, social stimulation from caregivers, and subsequent cognitive capabilities. Cross-cultural research designs also provided the basis for several major projects conducted by this group during the past year. In these studies the primary focus has been to specify the nature of infant-caretaker relations among infants living in different normal urban environments and infants living in different atypical environments, including traditional kibbutzim in Israel, middle-class homes in urban settings in Japan, France, and the U.S.A., and lower-class homes in Argentina and the U.S.A. The goals of these ongoing longitudinal studies are to identify and describe similarities and differences in common activities of mothers and infants in different cultures and to contrast the nature of basic early mother-infant interactions in different societies. Further, information is being obtained on assessing infant information-processing capabilities and on infant temperament.

During the past year data collection and analyses comparing the Japanese and U.S. mother-infant cohorts when the infants were 5, 13, and 20 months of age were completed. Different patterns of mother-infant interaction and different communicative styles of maternal speech were found between the two countries. The results also identified cultural-specific maternal behaviors and speech in each country. Analyses of maternal speech and mother and infant behaviors at 13 and 20 months have thus far uncovered activity and interaction patterns that are distinctive to these two disparate cultures as well as patterns that are similar between the two cultures, and which may point to processes universal in early development. Additionally, habituation (to mother's face and to a single familiar object) was studied from videotaped segments in each sample. These observations also indicate that habituation is a commonplace response of infants to faces and to objects naturally encountered in everyday settings. Moreover, the characteristics of habituation appear to be similar to those measured in the laboratory, and samples of Japanese and American infants appear to habituate similarly. Data collection for the other cross-cultural samples continued.

Detailed longitudinal study of patterns of mother-infant interaction provided the basis for several other major research projects in the CFRS this past year. A common objective of these studies has been to investigate both short- and long-term influences of caregiver behavior on cognitive development in infancy and early childhood, especially among individuals who exhibit different patterns of habituation during the first months of life. The rationale for this emphasis stems from the fact that before children are old enough to enter formal social learning situations, nearly all of their experiences stem directly from interactions they have with their primary caretaker. In these studies caretaker-child interactions can be conceptualized as falling into four distinct categories: nurturant, material, social, and didactic. Mother-infant interactions are typically videotaped for subsequent

microanalysis in both home and laboratory settings. In most of these studies the interaction sessions are conducted when the infants are 2, 5, 13, and 20 months of age.

This year, data analyses were completed on a large normative sample of infants and their caretakers. Mothers' encouragement of infants' attention and infant habituation were examined at 5 months in relation to toddlers' play, language comprehension, and representational competence at 13 months. Early maternal encouragement explains unique variance in language comprehension and representational competence after partialling habituation, and habituation predicts play, language comprehension, and representational competence after the influences of both early and later stimulation are partialled. These findings indicate that maternal stimulation predicts toddler cognitive ability over and above infant habituation, and also that links between habituation and cognition are not solely mediated by maternal stimulation.

In the same study, relations among toddlers' language production, language comprehension, play competence, and attention span at 13 months were examined with and without mothers' didactic encouragement partialled. Language production and flexible language comprehension systematically covary, and play competence systematically covaries with flexible language comprehension and with attention span. These relations maintain even when concurrent maternal stimulation is partialled. In addition, mothers' contingent responsiveness to their 5-month-olds predicts both representational competence (play and language comprehension) and exploratory competence, even after infant habituation and early and later maternal noncontingent stimulation are partialled. This finding suggests that contingent responsiveness in infancy uniquely predicts competencies at the start of the second year.

In assessment of mother and toddler interaction, mothers' demonstrations and elicitations of nonsymbolic and symbolic play were examined in relation to toddlers' nonsymbolic and symbolic play between 13 and 20 months. The relative sophistication of mothers' and toddlers' play closely parallel one another at both ages. Mothers are highly stable on all activities, whereas only nonsymbolic play in toddlers is stable. At both 13 and 20 months, individual differences among mothers and among toddlers are associated with one another in highly specific ways. Mothers' symbolic play relates to children's symbolic play, and their nonsymbolic play relates to children's nonsymbolic play. However, neither mothers' play nor toddlers' early play demonstrate any predictive influence on the later activities of the other.

The potential consequences of early disruptions in infant-caregiver interactions were seen in results of a retrospective study in which children's social, emotional, and cognitive competencies were assessed at 4 years of age and then examined with respect to the caregiving status of the mothers during the first few months of life. Preliminary analyses of data from the first 60 subjects reveal significant differences among children at 4 years of age who experienced varying modes of care in the first 6 months of life. For example, children cared for by their own mothers in the first 6 months of life have higher Adjustment scale scores than age-mates who experienced increased amounts of nonmaternal care in the company of other children in the first 6 months of life. Higher Adjustment scores indicate better performance on intellectual (cognitive and verbal) assessments and a lower number of disturbed behaviors, as indicated on the Preschool Behavior Questionnaire (PBQ), including hostile/aggressive, anxious, and hyperactive/distractible behaviors. In addition, children who, in the first 6 months of life, had less stable nonmaternal care (i.e., multiple caregivers and locations of care which translated into higher numbers of changes in their care histories) score significantly lower on the cognitive index and the perceptual/performance scale of the McCarthy Scales of Children's Abilities. These findings hold even when concurrent caretaking circumstances at 4 years and maternal education level are taken into account. These findings have important policy implications regarding the advisability of out-of-home care for infants prior to 6 months of age, and they have provided the motivation to carry out a large-scale prospective study of infants whose caretaking experiences vary systematically in their first few months of life.

Another longitudinal study investigating mother-infant interactions focused on specific aspects of emerging language capabilities and play activities at 13 and 20 months. Analyses of interaction patterns at 13 months revealed that maternal didactic interactions, but not maternal social interactions, relate independently to toddlers' language, not play, skills. Conditional associations between domains significantly augment explained variance in both toddler language and play. For example, social interaction is positively associated with language only in dyads where mothers, rather than toddlers, control object-oriented exchanges. Neither social nor didactic interactions are independently associated with toddler pretense play, but their combination is. Conditional relations between interactions and toddler skills suggest the importance of maternal modulation of social and didactic exchanges, depending on toddler attention and initiative. Longitudinal data were analyzed in order to uncover predictive relations between mother-toddler interaction at 13 months, toddler competence at 13 months, and toddler competence at 20 months. The results show strong continuities between toddler language performance at 13 and 20 months, but little correspondence between 13- and 20-month play performance.

Further examination of 13-month toddler data yielded an interesting and unexpected result: Despite the fact that all toddlers were evaluated within several weeks of their 13-month birthdays, subtle variation in toddler age is related to language comprehension assessed by two independent measures—a standardized instrument and a detailed maternal interview. The association of toddler age with language comprehension at such an early age, when age variation was quite minimal, is noteworthy. This finding has potential technical relevance to other researchers in the area of language development.

The Brain, Behavior, and Communication Section (BBCS), led by **David Symmes**, studies the production and utilization of vocal signals by group-living squirrel monkeys in terms of both the acoustical properties of the signals and their information content for group members. Parallel acoustical analyses are conducted on selected vocal patterns of other primate species, including humans. This past year, caregiver-infant interactions and patterns of social play also provided the basis for major research efforts in the BBCS. Longitudinal study of the emergence of vocal communication in socially reared squirrel monkeys revealed interesting qualitative changes in the nature and apparent target of vocal utterances displayed by infants during their first few months of life, especially when the mothers and infants are housed in small social groups instead of in isolated dyad cages. Isolated mothers average less than 1 call per hour to their infants in the first month of life, when youngsters are still on the mothers' backs almost continuously. In contrast, a mean of 81 calls per hour are directed to infants housed in a small group during month 1. 93% of these are from aunts, including those carrying their own babies; only 7% of these calls are from an infant's own mother. This ratio changes abruptly when infants begin to leave their mothers' backs in month 2 and 3: aunts' rates fall precipitously while mothers increase their rates to keep in contact with wandering infants. Almost all of the vocalizations directed to infants in the first 2 months are "caregiver calls," a type which shows many similarities to "motherese" in humans. Infants respond, either vocally or with vocalizations plus gestures, to 15-20% of caregiver calls in the first month, increasing to 30-50% in month 2. Particularly in the first month, most infant vocalizing is responsive, not spontaneous, under these low stress conditions. About 75% of infant utterances are made in response to caregiver calls directed to them at close range, usually by aunts. Infants (1-4 weeks old) in small groups emit an average of 19 calls per hour, compared to 7 per hour for infants housed along with their mothers.

Continued study of vocalizations emitted by juvenile squirrel monkeys during bouts of active play revealed that the primary function of "play" vocalization is to alert adult group members to be more vigilant when the young are absorbed in play. Play has been shown to be a risky activity in other primates, exposing the vulnerable young to predation. However, the protection afforded by adults monitoring such activity allows youngsters to play with abandon and in large numbers and compensates for what would otherwise be a maladaptive activity, where animals crash through the trees, vocalizing loudly and oblivious to predators. This finding is further evidence for both the importance of play and the degree and variety of indirect parental care in this species.

The Unit on Neuroethology, headed by John Newman, uses neuroscience techniques to study brain mechanisms involved in the production of various types of primate vocalizations by squirrel monkeys and to examine subtle acoustical differences in characteristic calls among several primate species. One study carried out in the Unit on Neuroethology focused on the vocal patterns of wild-living monkeys, in this case a troop of free-ranging squirrel monkeys in Costa Rica. The objectives of this study were to characterize the relationship between troop activity, spatial distance between troop members, and vocal patterns. Twenty-one adult female squirrel monkeys were marked with Nyanzol dye, facilitating individual identification. After a 3-week period of habituation, vocalizations were recorded onto a tape recorder along with ongoing activity of the vocalizer, distance to the nearest adult female, and troop activity. Subsequent analysis in the laboratory consisted of producing sound spectrograms for each vocalization of suitable quality, classifying the vocalization as to subtype (peep, twitter, smooth chuck, bent mast chuck), and assigning a unique code according to its place in a recording bout. Each call was then associated with the concomitant behavior of the vocalizer and distance to the nearest adult female. Statistical analysis revealed that distance of the vocalizer to the nearest adult female is a highly significant factor affecting rate of calling. For "peeps," the category containing the species-specific isolation call, there is a significantly greater incidence of these sounds at distances greater than 1 meter from the nearest adult female, whereas other vocal subtypes show no significant correlation with production rate and distance from the nearest neighboring female. No significant association was found between behavior of the vocalizer (foraging vs nonforaging or stationary vs traveling) and type of vocalization.

In a collaboration with the BBCS and the Caribbean Primate Research Center, vocalizations collected from juvenile rhesus monkeys briefly separated from their natural troop during routine veterinary examinations were analyzed using the BBCS computer system. A major finding was that the peak frequency and duration of "coo" calls changes systematically throughout the 24-hour period of separation. Moreover, the relative values of several vocal parameters of these calls also are systematically related to levels of plasma cortisol and ACTH, as well as lymphocyte counts in blood samples obtained during the separation.

Another study completed this year focused on the role of catecholamines in mediating isolation call production in adult male squirrel monkeys. In this study, conducted in collaboration with the Laboratory of Clinical Science, NIMH, peripherally administered drugs known to selectively inhibit either the A or B form of monoamine oxidase (MAO) were tested over a 5-fold dose range on production of the species-typical isolation call of squirrel monkeys ("isolation peep," IP). In contrast to 2 drugs with MAO-B inhibitory activity (milacemide and 1-deprenyl), the MAO-A selective drug clorgyline produces a dose-related decrease in IP production, but fails to completely suppress IP production even at the highest dose employed (5 mg/kg), and likewise fails to produce a significant decrease in locomotory activity. These results suggest that the catecholamine substrates of endogenous MAO-A enzyme (e.g., noradrenalin and serotonin) are likely to play a more central role in regulating IP production relative to other aspects of behavioral arousal such as locomotion. The substrates of endogenous MAO-B, on the other hand, appear to be less directly involved in regulating vocal behavior.

An ongoing project showing considerable progress this year involved the longitudinal assessment of the vocalizations of rhesus macaques subjected prenatally to a cerebro-ventricular shunt to correct experimentally induced hydrocephalus, done in collaboration with Drs. Suomi and Michejda (Georgetown University Medical School). Additional experimental and age-matched control subjects were studied, with a continued affirmation of the differences reported last year (experimentals producing more noisy and variable calls during brief social separation). More detailed analysis this year has revealed that there is a significant gender difference in the incidence of noisy vs tonal separation calls in the control animals, with females producing a greater proportion of noisy calls. By separating male and female experimentals prior to statistical analysis, it was shown that female experimentals are not statistically different from their control counterparts, whereas

male experimentals produce significantly less tonal and significantly more noisy calls ("leap-coos") than control males. Increased noise and variability has been shown to be a valid measure of increased distress or agitation in human infants, and may be an indication in the present study that females (and the experimental males) are more agitated when socially separated than are control males. NMR scans performed on all of the experimental animals failed to reveal any abnormalities in size or shape of the cerebral ventricles. However, a potential neural substrate for these vocal differences has been identified in a collaborative study with Dr. Bachevalier of the Laboratory of Neuropsychology, NIMH, in this case involving the longitudinal assessment of vocalizations in rhesus macaques receiving bilateral ablation of the inferotemporal cortex. Analysis of recordings made over the first month following surgery revealed that the vocal behavior of the experimentals is very similar to that of the corrected hydrocephalus animals, with both male and females that had been subjected to inferotemporal ablations showing reduced tonal and increased noisy calls during brief periods of social separation. As with the corrected hydrocephalus subjects, it is the males that show a significantly different ratio of tonal and noisy calls in comparison to age-matched controls of the same sex.

The Unit on Parent and Infant Studies, headed by **Frank Pedersen**, carries out investigations of parent-infant relationships. This year, in collaboration with the Unit on Neuroethology, the group conducted systematic analyses of human infant vocalizations as part of a large, longitudinal project. This project, for which data collection was completed, investigated emotional factors ascertainable in parents during the pregnancy period, reactivity to infant cries that have varying degrees of aversiveness, and the unique individuality of the infant as factors that collectively influence the course of the parent-infant relationship in the first year of life. First-time expectant mothers and their spouses were studied prior to and immediately following the infants' birth, and parents and infants were followed at 3, 9, and 12 months. Nonpregnant women were also studied with a subset of the procedures at times corresponding to the prenatal and 3 month periods. The study employed multiple measurements, including observational, self-report, and physiological procedures. Preliminary analyses, particularly for the prenatal and 3 month periods, have yielded several important findings.

First, physiological reactivity, as assessed by changes in heartrate, to the mildly stressful stimuli of recorded infant cries was attenuated among pregnant compared to non-pregnant women, even though both groups were generally similar in their subjective ratings of cries with differing degrees of aversiveness. On subjective ratings there were highly significant discriminations of the cries into two groups, one very aversive and one relatively nonaversive. Pregnancy status had no effect. On physiological measures of reactivity (heart rate change to the cry stimuli), the aversive cries elicited a pattern of response classically identified as more stressful than did the nonaversive cries. Independently of cry type, pregnant women showed a less reactive pattern after the first few seconds than was evident for the nonpregnant women. Vagal tone, a measure of heart rate variability that provides an independent measure of stress reactivity, was found to be in the lower range for a greater proportion of pregnant women than for the nonpregnant group. This suggests that pregnancy normally results in some attenuation of reactivity of the autonomic nervous system. Pregnant women whose vagal tone was not in the lower range also scored significantly higher in their reports of anxiety symptoms, suggesting that dampened reactivity may have a protective influence during fetal development.

A second set of findings relates to infant individuality. In connection with the neonatal assessments, brief recordings of the infant's cry were obtained. Cry samples were also obtained at age 3 months. These samples (N = 36) were analyzed for eight acoustic characteristics. Four acoustic parameters were significantly correlated between the neonatal and 3-month period, indicating stable individual differences. Three of these dimensions were significantly related to maternal ratings of infant temperament. For example, a high fundamental frequency in the cry was related to parental ratings of the infant being unadaptable, unpredictable, and having a "difficult" temperament. These results extend into

the normal range some of the conclusions that have previously been drawn with clinically significant groups of infants.

Other results bearing on infant individuality were found in relation to the measure of heart rate variability, vagal tone. Infants whose baseline vagal tone at age 3 months was in the higher range were found in a laboratory procedure to have a different visual fixation pattern that was consonant with more rapid processing of visual stimuli. They also were rated in the laboratory procedure as more soothable, and fewer infants in the high vagal tone group required soothing interventions. Moreover, ratings of negative affect in these infants were significantly lower. As in the findings involving cry acoustics, these results suggest there is a biological underpinning to infant temperament.

The final set of results relate to a new questionnaire procedure developed for this project. This measure, self-efficacy in a nurturing role, was found to have good internal psychometric properties (alpha coefficients in the .80s), good reliability over a 5 month period, which includes the vicissitudes of the birth of a baby  $r = .63$  for mothers), and meaningful but moderate overlap with a more generalized measure of the mother's emotional state, her anxiety level  $r = -.41$ , prenatal period and  $r = -.49$ , postnatal period). The mother's accrual of experience with her baby was associated with a significant increase in self-efficacy in her nurturing role ( $p < .001$ ), but her anxiety level appeared largely unchanged. One interesting hypothesis that remains to be tested is whether self efficacy relates to the mothers' reactivity patterns to the cry stimulus.

Analyses of data collected on both parents and infants through the end of the first year of life are currently underway. Although the Unit on Parent and Infant Studies will be formally disbanded with the retirement of Dr. Pedersen, Drs. Huffman and del Carmen will continue the data analyses under the auspices of the LCE's Office of the Chief.

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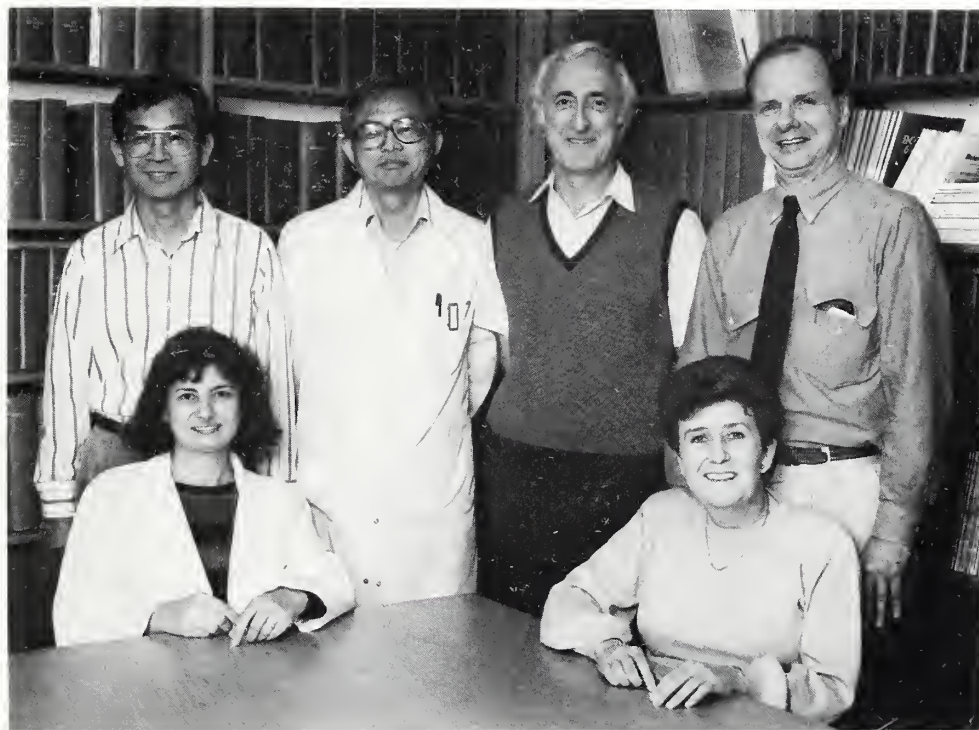
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## ENDOCRINOLOGY AND REPRODUCTION RESEARCH BRANCH

**Kevin J. Catt, M.D., Ph.D., Chief**



Left to right: K-P. Huang, G. Aguilera, H-C. Chen, K. Catt, M. Dufau, C. Strott

The research programs of the Endocrinology and Reproduction Research Branch are directed at the elucidation of cellular mechanisms involved in hormone secretion and action. These programs include studies on the characterization of peptide hormones and their cellular receptors; the mechanisms of peptide hormone action in endocrine target cells; the structure-function relationships of peptide and glycoprotein hormones; and the regulation of hormone biosynthesis and secretion. Of particular interest are pituitary-gonadal and pituitary-adrenal regulation, the control of ovarian activity during the reproductive cycle and pregnancy, and the receptor-mediated control of pituitary, gonadal, and adrenal function. During the current year, research has been performed on the receptors and signalling processes that are responsible for the control of secretory responses and differentiation in endocrine target cells. The role of hormones in cellular regulation has also been examined in selected forms of normal and disordered human endocrine function, and in appropriate animal model systems for the analysis of hormone secretion and the stimulatory and inhibitory control of target-cell function. The staff of the ERRB share common interests in the mechanisms of action of peptide and glycoprotein hormones, the role of neuropeptides in hypothalamic-pituitary regulation and stress, the control of gonadal and adrenal function by pituitary hormones, the renin-angiotensin system and aldosterone secretion, and the mechanisms and roles of protein phosphorylation in metabolic regulation.

The Section on Hormonal Regulation, led by Kevin Catt, performs research on the control of endocrine target cells by peptide hormones, in particular the characterization, regulation, and activation mechanisms of membrane receptors for gonadotropin-releasing hormone (GnRH), angiotensin II (AII), and gonadotropins. The receptor-mediated actions of hypothalamic releasing peptides and other regulators of pituitary hormone secretion are studied in cultured anterior pituitary cells. The actions of angiotensin II are investigated in

cultured adrenal glomerulosa cells, and those of gonadotropins are analyzed in ovarian granulosa and luteal cells.

Pituitary gonadotropin secretion is primarily regulated by GnRH, which binds to and activates specific receptors in the plasma membrane of the gonadotroph. The GnRH receptor has been solubilized in active form with non-ionic detergents and shown to exist as a 250 kDa complex under non-denaturing conditions. This large complex contains a 52 kDa component, which is demonstrable by photo-affinity labeling, and which corresponds to the GnRH binding subunit of the receptor. GnRH action in pituitary gonadotrophs is expressed through the activation of calcium-phosphoinositide signaling mechanisms, leading to a characteristic biphasic profile of cytosolic calcium elevation and gonadotropin release from the pituitary gland. The initial phase of LH secretion has been found to depend largely on mobilization of intracellular calcium, whereas the sustained phase of hormone secretion is dependent on continued entry of extracellular calcium through plasma-membrane channels. In addition to the  $\text{InsP}_3$ -mediated early rise in  $[\text{Ca}]_i$  and LH release, other phospholipid products including diacylglycerol (DG) and arachidonic acid (AA) are involved in cellular activation by GnRH. Studies with enzyme inhibitors revealed that GnRH activates two distinct lipases, DG lipase and phospholipase  $A_2$ , to produce arachidonic acid, which is metabolized to hydroxyeicosatetraenoic acid derivatives and leukotrienes in pituitary cells. Activation of protein kinase C (PKC) by DG also participates in GnRH action, in part by mechanisms independent of calcium mobilization, but also by promoting calcium influx through dihydropyridine-insensitive plasma-membrane channels. The involvement of PKC in gonadotropin synthesis and secretion was demonstrated in pituitary cells depleted of PKC, in which the secretory actions of GnRH and phorbol esters are substantially impaired. These findings indicate that PKC participates in both the acute and long-term regulatory actions of GnRH in pituitary gonadotrophs, by enhancing calcium entry during the secretory response to agonist stimulation, and also by increasing gonadotropin synthesis during prolonged exposure to GnRH.

Studies on the nature of the calcium channels involved in pituitary hormone secretion revealed the presence of L-type voltage sensitive calcium channels (VSCC) in all five populations of anterior pituitary cells, with an average number of about 1000 channels per cell. In gonadotrophs, calcium entry through L-type VSCC was found to account for about 50% of the elevated cytosolic calcium and LH secretory response during sustained stimulation by GnRH. Furthermore, marked inactivation of VSCC during GnRH action was shown to occur by a calcium-dependent mechanism and to be accompanied by a corresponding decrease in LH responsiveness to GnRH. This process of calcium channel inactivation is responsible for the onset of desensitization, a prominent feature of GnRH action and the cellular basis of therapeutic suppression of gonadotropin secretion by treatment with GnRH antagonists.

In addition to the well-recognized form of hypothalamic regulation by GnRH, gonadotrophs were found to be more slowly activated by neurohypophyseal hormones. This action is mediated by a distinct receptor of the oxytocin type, in contrast to the vasopressin-type receptor that has been shown to mediate ACTH release in pituitary corticotrophs. Oxytocin may provide a complementary hypothalamic control mechanism for the long-term modulation of gonadotropin secretion, and may contribute to the basal level of LH secretion, upon which the pulsatile action of GnRH is superimposed to produce the normal physiological profile of episodic gonadotropin release.

The structural properties and activation mechanisms of the angiotensin II (AII) receptor were studied in bovine adrenal glomerulosa cells and other target tissues. The adrenal AII receptor was purified by affinity chromatography and subjected to microsequencing, and cDNA probes were prepared and applied to the screening of an adrenal cDNA library. A further approach to cloning the AII receptor was initiated by screening mRNA, prepared from an adrenal cDNA expression library cloned into phage lambda Zap, in the *Xenopus* oocyte. The detection systems employed in this approach were based on the measurement of electrophysiological and light responses (measured by the photoprotein, aequorin) in oocytes

injected with mRNA from adrenal extracts and the expression library. The aequorin detection system was established and validated by measuring the light responses elicited by ligand-induced calcium mobilization in *Xenopus* oocytes injected with poly (A)<sup>+</sup> mRNA from rat adrenal cortex and brain, during stimulation by AII, acetylcholine and glutamate. This system is currently being used to detect angiotensin II receptor mRNA in the phage expression library, and to analyze the properties of endogenous and exogenous AII receptors in the oocyte.

Studies on the mechanism of action of AII, performed in cultured bovine adrenal glomerulosa cells, showed that the rapid effect of AII on Ins-1,4,5-P<sub>3</sub> formation is accompanied by elevation of cytosolic calcium during the first minute of stimulation, with peaks in both InsP<sub>3</sub> and [Ca<sup>2+</sup>] responses at 5-10 sec. The subsequent plateau in calcium is associated with secondary increases in Ins-1,4,5-P<sub>3</sub> and Ins-1,3,4,5-P<sub>4</sub>, suggesting that both inositol phosphates participate in the maintenance of the sustained calcium response to agonist stimulation. Extending the Section's previous studies on the production and metabolism of inositol phosphates in AII-stimulated adrenal glomerulosa cells, the conversion of Ins-1,3,4,6-P<sub>4</sub> to inositol pentakisphosphate (InsP<sub>5</sub>) was demonstrated in bovine adrenal cytosol and permeabilized bovine glomerulosa cells, establishing Ins-1,3,4,6-P<sub>4</sub> as a link between agonist-stimulated InsP<sub>3</sub> metabolism and the higher inositol phosphates (InsP<sub>5</sub> and InsP<sub>6</sub>), recently detected in the adrenal gland and other mammalian tissues. In addition to the conversion of Ins-1,3,4,6-P<sub>4</sub> to InsP<sub>5</sub>, two other InsP<sub>4</sub> isomers were detected and characterized in AII-stimulated adrenal cells. These were identified as Ins-3,4,5,6-P<sub>4</sub>, shown to be an additional and major precursor of InsP<sub>5</sub>, and its stereoisomer, Ins-1,4,5,6-P<sub>4</sub>, both of which were found to be formed by dephosphorylation of InsP<sub>5</sub>. AII stimulation was also found to cause a short-term increase in conversion of InsP<sub>5</sub> to Ins-1,4,5,6-P<sub>4</sub>, the first evidence for agonist-regulated control of InsP<sub>5</sub> breakdown. In addition, AII causes a progressive elevation of Ins-3,4,5,6-P<sub>4</sub> and InsP<sub>5</sub>, thus providing further evidence for a connection between agonist-induced phosphoinositide hydrolysis and the production of higher inositol polyphosphates in the adrenal glomerulosa cell. These findings provide compelling evidence for the existence of a major contribution from the metabolites of Ins-1,4,5-P<sub>3</sub>, the primary signaling molecule responsible for calcium mobilization, with the slowly formed higher inositol polyphosphates, compounds for which alternative biosynthetic pathways may also exist via intermediates of as yet undetermined origin.

Previous investigation of the molecular basis of hormone action during granulosa cell differentiation included evaluation of the functions and mechanisms of action of pituitary hormones and growth factors. In such studies, TGF- $\beta$  was found to exert bifunctional actions on the maturation of granulosa cells, and to modulate FSH-induced stimulation of cAMP formation, steroidogenesis, and LH receptor expression. TGF- $\beta$  also accelerates the maturation of both follicle-enclosed oocytes and cumulus-oocyte complexes, with significant increases in the rate of germinal vesicle breakdown. Other growth factors, including IGF-I, IGF-II, and EGF, also stimulated germinal vesicle breakdown. For further studies on growth control of rat granulosa cells, which do not undergo division in culture, a continuous cell line was derived by transformation of granulosa cells with an SV40 tsA255 mutant, which has a temperature-sensitive mutation in the gene required for maintenance of transformation. This granulosa cell-derived line (RGA-41S) exhibits temperature-regulated proliferation behavior with unrestrained growth and a transformed phenotype at the permissive temperature (33 °C) and a differentiated non-transformed phenotype with growth arrest at the nonpermissive temperature (40 °C). The cultured RGA-41S cells were found to produce IGF-I mRNA and to secrete IGF-I, and also to express receptors for IGF-I as well as the corresponding mRNA. These cells provide a model system for studies on the paracrine and autocrine actions of IGF-I in the growth and differentiation of ovarian and other epithelial cells.

In normal granulosa cells studies were begun on the regulation of the ovarian renin-angiotensin system, employing solution hybridization procedures to measure the steady state levels of renin and angiotensinogen mRNA during maturation and stimulation of granulosa cells by gonadotropins and growth factors *in vitro*. Also, based on recent findings that

growth hormone releasing hormone (GHRH) increases the incidence of ovulation in women undergoing gonadotropin therapy for infertility, and that immunoreactive GHRH is present in the gonads, local actions of GHRH on granulosa cell function were evaluated *in vitro*. In initial studies, GHRH exerted consistently stimulatory effects on cyclic AMP production and steroidogenesis in the granulosa cell, revealing a local regulatory mechanism with the potential to synergize with FSH at the ovarian level. These studies are being extended to include characterization of the GHRH receptor of the ovary and its relationship with receptors known to mediate the actions of VIP and related peptides upon ovarian function.

The Section on Endocrine Physiology, led by Greti Aguilera, investigates physiological and pathological aspects of circulatory homeostasis and neuroendocrine regulation, including mechanisms of adaptation to stress. This program also includes studies on the role of the renin-angiotensin system in the regulation of mineralocorticoid secretion and blood pressure, and the effects of AII in other systems including the pituitary and gonads. A major purpose of this project is to analyze the physiological functions of the renin-angiotensin system, including the effects of AII in circulatory homeostasis, fetal development, and control of pituitary and gonadal function.

AII mediates the increase in aldosterone secretion during sodium restriction, but the adrenal effects of the peptide depend on the sensitivity of the glomerulosa zone to AII. Previous studies in the rat have demonstrated that the adrenal responsiveness to AII depends on trophic effects of the peptide and on the modulatory effect of other regulators, such as dopamine, atrial natriuretic factor (ANF) and somatostatin. Elucidation of the mechanisms by which these factors modulate the effects of AII requires a precise knowledge of the mechanism of action of AII. The cellular response to AII in the adrenal glomerulosa zone includes calcium mobilization and phospholipid turnover, with possible activation of PKC. However, the relative importance of these events in stimulation of steroidogenesis is not understood. This is particularly unclear in regard to PKC, since phorbol esters, known stimulators of the enzyme, are ineffective in stimulating aldosterone production under most experimental conditions.

The role of PKC in the steroidogenic action of AII was investigated by depleting endogenous PKC during prolonged incubation of glomerulosa cells with phorbol ester. Direct measurement of PKC in rat glomerulosa cell cytosolic and detergent-solubilized membrane fractions, purified by DEAE cellulose chromatography, showed that basal PKC activity is 2 to 3-fold higher in cytosol than in membranes. After incubation of the cells with AII for up to 60 min, PKC activity in the cytosol progressively decreases by up to 27%, while the membrane shows a transient increase of 15% at 15 min, returning to basal by 60 min. Incubation of cells with 100 nM 12-0-tetradecanoyl-phorbol-13-acetate (TPA) results in transient translocation of PKC activity to the membrane (15 min), which is followed by a 70 to 90% decrease in total cellular enzyme activity after 2 hr. In PKC-depleted cells, the aldosterone response to ACTH is increased by 25%, but AII-stimulated steroidogenesis remains unchanged. In contrast, in cells in which PKC is translocated to the membrane by 15 min preincubation with TPA, the aldosterone response to AII is enhanced by 40% while the response to ACTH is reduced by 30%; under these conditions membrane PKC levels rapidly return to basal. However, the changes in aldosterone response are still evident when addition of AII or ACTH is delayed for up to 30 min after removal of TPA, indicating a persistent modification in the cell membrane secondary to PKC activation. Aldosterone responses to potassium are not altered by preincubation of the cells with TPA. The inactive phorbol ester analog, 4 $\alpha$ -hydroxyphorbol-12,13-dibutyrate, has no effect on steroid responses to either stimulus. The small but significant translocation of PKC activity from cytosol to membrane following treatment of rat adrenal glomerulosa cells with AII suggests that AII activates PKC. However, the fact that aldosterone responses to AII are potentiated during TPA-induced PKC translocation to the membrane indicates that AII and phorbol esters do not share the same mechanism of action in the regulation of steroidogenesis. In addition, the full aldosterone response to AII, despite marked cellular PKC depletion following prolonged preincubation with TPA, argues against the involvement of PKC in the stimulation of aldosterone produc-

tion by AII. It is possible that activation of PKC by AII mediates effects other than steroidogenesis, such as trophic maintenance and/or growth of the adrenal glomerulosa cell.

The distribution and role of AII receptors during early and late embryonic development was further studied in whole mouse blastocysts and membrane-rich fractions from rat conceptuses. In early mouse embryos there is no detectable AII binding, but late gestation rat fetuses show specific and high affinity binding, and the concentration of AII sites is similar to membranes from whole and eviscerated fetuses. Scatchard analysis of the binding data indicate  $K_d$  values ranging between 0.7 and 0.9 nM. Binding is first detectable at day 10 of fetal life and increases progressively with gestational age. AII receptors in skin and skeletal muscle membrane show an 80% decrease in AII receptors one day after birth, and subsequently become almost undetectable in the adult. The functional significance of these binding sites depends on the availability of the agonist ligand, angiotensin II. Immuno-reactive AII-like material, with chromatographic properties identical to an AII standard, can be detected in 10-day fetuses and was shown to be bioactive in an AII receptor assay, providing evidence for potential activation of the fetal receptors by the cognate ligand AII.

Localization of renin mRNA in fetal tissues was analyzed by *in situ* hybridization, using an antisense  $^{35}\text{S}$ -labeled riboprobe transcribed from a mouse renin 2 cDNA clone. Hybridized tissue sections show high intensity staining in the fetal kidney and adrenal cortex. Northern blot analysis confirmed the expression of high levels of renin mRNA in the fetal kidney. However, only low and inconsistent labeling is observed in the epidermis. The physiological effects of AII in the fetus were investigated in cultured fibroblast-like cells from fetal skin, which contain abundant AII receptors with properties identical to those described in fetal membranes. Stimulation of the cultured cells with AII results in increases in cytosolic calcium and inositol phosphate formation, indicating that the binding sites correspond to receptors linked to intracellular responses. The abundance, timing of expression, and unique localization of functional AII receptors in the fetus suggest a role for AII in fetal development.

Studies on the hypothalamic-pituitary system have focused on the properties and regulation of corticotropin releasing factor (CRF) receptors, and the mechanisms of interaction between CRF and other regulators of ACTH secretion, with emphasis on adaptation to stress. The biphasic ACTH responses to prolonged immobilization were previously shown to be accompanied by decreases in pituitary CRF receptors, and by hypersensitivity of the pituitary to CRF infusion and to a second novel stress. Extension of these studies using a more mild form of stress, intermittent immobilization for 2.5 hr daily, showed complete desensitization of the ACTH response after the first 4 days of immobilization, while plasma glucocorticoids are markedly increased. Despite a decrease in pituitary CRF receptors, ACTH responses to a novel stress, but not to CRF infusion, are enhanced. These responses are similar to those observed in types of human depression, in which hypercortisolemia is a characteristic feature. CRF receptors in the intermediate pituitary are unchanged during immobilization stress. In contrast to the changes during immobilization stress, CRF receptors are unaltered in the anterior pituitary during cold stress, but are markedly increased in the intermediate lobe. The binding characteristics of CRF receptors, determined in membranes from the neuro-intermediate lobes, are almost identical to those in the anterior pituitary. Analysis of the solubilized cross-linked CRF-receptor complex in different target tissues by gel electrophoresis showed similar properties in both pituitary lobes, with a molecular weight of about 70 kDa. It is known that CRF receptor occupancy results in activation of adenylate cyclase. In addition, experiments showing that pharmacological inhibition of PKC inhibits ACTH release suggest that the CRF receptor may also be linked to a calcium/phospholipid-dependent transduction system.

The Section on Molecular Endocrinology, led by Maria Dufau, investigates the molecular basis of peptide hormone action, with particular emphasis on the characterization of gonadotropin receptors, activation of steroid biosynthesis in gonads and adrenal, and analysis of the biological activity of circulating gonadotropins. A major aspect of this program is the

characterization of gonadal gonadotropin and prolactin receptors, and of the physical and functional relationships of the LH receptor site with adenylate cyclase.

The Nb2 lymphoma cell depends on lactogens for proliferation, and provides an invaluable model for the study of prolactin (PRL) receptor coupling functions and of messengers for prolactin's multiple biological actions. Recent studies in the Section demonstrated that the proliferative effect of prolactin is modulated by pertussis and cholera toxins, and that these changes are independent of the effects of cyclic AMP on prolactin-induced mitogenesis, indicating that G-proteins may be involved in signal transduction steps. It was also found that exposure of Nb2 cells to prolactin causes time- and dose-dependent changes in the ability of specific 38 kDa and 41.5 kDa membrane proteins to be subsequently ADP-ribosylated by pertussis and cholera toxins, respectively. These include rapid reduction of the 41.5 kDa substrate for cholera toxin and a marked increase of the 38 kDa substrate for pertussis toxin. The early changes observed in cholera toxin-induced ADP-ribosylation suggest a rapid and direct effect at the membrane level, presumably involving the interaction of prolactin with its receptor, dissociation of the  $\alpha$ -subunit from the  $\beta\gamma$ -complex of the trimeric G-protein and activation of the  $\alpha$ -subunit. This may account for the reduced susceptibility to ADP-ribosylation after hormone treatment, as suggested in certain other systems. The changes revealed by pertussis toxin may result from structural changes of the putative G protein caused by interaction with the activated lactogen receptor, and/or may involve preferential increases in toxin-sensitive G-protein synthesis. This study provides the first demonstration of a direct effect of prolactin on G-proteins, and suggests that such regulatory proteins are involved in the signal transduction mechanism by which occupancy of the prolactin receptor leads to proliferation of Nb2 lymphoma cells. The influence of prolactin on the G-proteins of Nb<sub>2</sub> lymphoma cells has opened an avenue for elucidation of lactogen receptor-coupled functions in mitogenesis, an approach that may have general application for signalling studies in other cells expressing the diverse actions of prolactin in its several target tissues.

The purified ovarian LH/hCG receptor was identified as a monomeric protein ( $M_r$  80,000) and its homogeneity was confirmed by microsequencing. The N-terminal peptide sequence is NH<sub>2</sub>-R-E-L-S-G-S-R-X-REP-D-X-D-X-A-P-D-G. A sequence derived from cyanogen bromide cleavage (P-L-V-G-I-S-N-Y) was used to raise antibodies and to screen genomic and cDNA libraries. A cDNA clone was isolated with 61% overall nucleotide similarity with the recently cloned LH receptor, and the possibility that this molecule is a yet unidentified receptor or channel is under investigation. The purified testicular and ovarian LH/hCG receptors were shown to be phosphorylated *in vitro* by the catalytic subunit of cAMP dependent protein kinase. The influence of hormone on phosphorylation is related to stages of trophic hormone binding ("early" loose binding and "late" tight binding), i.e., the rate of phosphorylation is controlled by conformational changes. Since the LH/hCG receptor of testis and ovary is only available in microgram quantities, the more stable phosphorylated receptors were employed to facilitate structural studies. In initial studies using reduced phosphorylated receptor, it was demonstrated that the LH/hCG receptor contains sialylated N-linked carbohydrate chains of the biantennary type, the hybrid type or both, and that glycosylation can account for the size difference of ovarian and testicular receptors. Conditions were established for complete enzyme digestion (neuraminidase, N-glycanase) of phosphorylated receptors, conditions that also preserve hormone binding activity in the native blotted receptor. Studies on blotted receptors revealed components with  $M_r$  from 90-92 kDa to 82 kDa (testis) and 80-85 kDa (ovary), with a decrease to 60 kDa in both tissues after N-glycanase treatment. Subsequently, studies on the binding of <sup>125</sup>I-hCG to blots of desialylated or deglycosylated native receptor after SDS/PAGE demonstrated that the terminal sialic acid of the glycosidic chain is not involved in hormone binding. However, the removal of glycosyl residues by N-glycanase causes a major decrease in hormone binding, indicating the participation of N-linked carbohydrates in agonist-receptor interactions.

Luteinizing hormone is the major regulator of Leydig cell differentiation and steroidogenic function. A number of hormones produced by the Leydig cell (e.g., estrogen, angiotensin, CRF, vasopressin) and the tubular compartment (inhibin, TGF- $\beta$ ), can influence both acute

and chronic actions of LH. Conversely, hormones produced in the Leydig cells modulate tubular function (e.g., androgen,  $\beta$ -endorphin, and oxytocin). The stimulatory actions of LH on Leydig cell function can be negatively influenced by the effects of AII, which are exerted through the guanyl nucleotide inhibitory unit of adenylate cyclase. Recent studies in the Section have revealed a novel inhibitory action of CRF through specific high-affinity/low-capacity receptors in the Leydig cells. This process involves a pertussis toxin insensitive guanyl nucleotide regulatory unit with interaction between signalling pathways, which results in inhibition of LH-induced cAMP generation and consequently of steroidogenesis. In contrast to its actions in brain, pituitary and other peripheral tissues, CRF receptors in the Leydig cell are not coupled to Gs and stimulation of adenylate cyclase. Rather, the inhibitory action of CRF in the Leydig cells is exerted through direct or indirect activation of PKC, at the level of one or more components of the adenylate cyclase system. These studies have demonstrated a novel mechanism for the action of CRF, probably mediated by a CRF receptor subtype, and have highlighted the importance of interactions between different signal transduction pathways in cellular homeostasis. Also, these observations have demonstrated that CRF has potent antireproductive effect at the testicular level. Since CRF is synthesized in the testis and is present in Leydig cells, it is likely that locally produced CRF exerts negative autocrine modulation on the stimulatory action of LH on Leydig cell function.

Physiological increases in endogenous LH action cause positive regulation of membrane receptors and steroidogenesis, while major elevations in circulating gonadotropin can induce down-regulation of LH receptors and desensitization of steroid responses in the adult cell. Gonadotropin-induced desensitization in adult rat testes includes an estrogen-mediated steroidogenic lesion of the microsomal enzyme(s)  $17\alpha$ -hydroxylase/ $17$ - $20$  desmolase. For further understanding of the structure and regulation of this key enzyme of the androgen pathway, the rat P450- $17\alpha$  cDNA was cloned and sequenced. This cDNA expresses  $17\alpha$ -hydroxylase/ $17$ , $20$  desmolase activities when transfected into COS cells. From the deduced amino acid sequence, two transmembrane regions were identified. The first of these is a signal peptide for insertion in the endoplasmic reticulum, and the second, which is separated from the first transmembrane region by 122 amino acids, may serve as the stop-transfer sequence. The carboxyterminal non-transmembrane region is composed of 4 hydrophobic clefts, of which cleft II is likely to contain the putative steroid binding site for both hydroxylase and lyase activities. Gonadotropin stimulation and desensitization of P450- $17\alpha$  dependent enzymes ( $17\alpha$ -hydroxylase/ $17$ , $20$  desmolase) are related to the levels of P450- $17\alpha$  mRNA, which show concomitant increases and decreases during the stimulatory and desensitization phases of LH action in the Leydig cell.

LH is released from the pituitary gland into the circulation as episodic pulses of high biological activity. This episodic mode of secretion may serve to overcome gonadotropin-induced desensitization of hormone-responsive gonadal cells. The exact nature of glandular secretory events is difficult to discern *in vivo*, since underlying patterns of hormone release are confounded by the effects of metabolic clearance. Using a deconvolution model that allows the calculation of endogenous clearance kinetics and secretory rates simultaneously, multiple parameter deconvolution disclosed an endogenous bioactive LH half-life of  $53 \pm 5.4$  min and an endogenous production rate of  $0.48 \pm 0.06$  mIU/ml/min. The bioactive LH secretory burst, with half-duration of  $12.2 \pm 1.5$  min, occurs at intervals of  $56 \pm 1.3$  min and achieved amplitudes of  $2.1 \pm 0.26$  mIU min. LH secretory bursts are positively correlated with the duration of the subsequent interpulse interval. Application of the deconvolution algorithm, in conjunction with the rat Leydig cell testosterone bioassay, reveals the presence of distinct burst-like secretory events acted on by exponential metabolic clearance mechanisms, and provides a good model for analysis of bioactive plasma hormone concentration profiles *in vivo*. Such multifaceted changes might not be readily apparent using deconvolution techniques that require known half-lives and/or conventional pulse analysis of plasma hormone concentration. The multiple parameter deconvolution approach may help to unmask pathophysiological states with alterations in endogenous hormones and their clearance *in vivo*.

In related studies, the time-dependent effects of estrogen on the kinetics of GnRH's self-priming action on the pituitary gland were analyzed in post-menopausal women on intravaginal estradiol replacement therapy. The self-priming action of GnRH on bioactive LH release is maximal after 5 to 10 days of estrogen exposure, and can be defined as a percentage increase in the amplitude of a second peak of bioactive LH provoked by exogenous GnRH (10 micrograms, i.v., given at 2-hourly intervals) compared with the first LH peak. These findings indicate that estrogen regulates the human pituitary gland's responsiveness to GnRH, such that enhanced secretion of biologically active gonadotropin occurs during serial exposure to GnRH stimulation. The finding of GnRH self-priming induced by estradiol is of importance to an understanding of possible mechanisms governing the generation of the preovulatory gonadotropin surge.

The Section on Adrenal Cell Biology, under the direction of **Charles Strott**, investigates the physiology and regulation of adrenal steroidogenesis, by characterizing cellular steroid binding proteins and soluble factors that mediate steroidogenic responses to ACTH, and by analyzing cellular mechanisms of cholesterol utilization in steroid biosynthesis. The Section is also interested in the development of adrenocortical zonation and the regulation of adrenal steroidogenesis, and is currently concentrating on two areas of research: 1) adrenocortical calmodulin, calcium- and calmodulin-binding proteins, protein kinase systems, and the post-translational modification of proteins; 2) purification, immunology, and functional activity of soluble and membranous adrenocortical proteins, including steroid-binding proteins.

The molecular mechanisms involved in the action of ACTH on the adrenal cortex are only partly understood. What is known is that ACTH binds to a cell surface receptor and stimulates adenylate cyclase, leading to an increase in intracellular cAMP and the activation of cAMP-dependent protein kinase. The steroidogenic response to ACTH can be separated into acute and subacute aspects. The acute response (sec-min) occurs primarily at the level of mitochondria, and involves the translocation of cholesterol to the inner mitochondrial membrane followed by the conversion of cholesterol to pregnenolone (rate-limiting). The subacute response (hours) appears to occur at the level of the genome, and involves synthesis of enzymes and co-factors. Both the acute and subacute responses to ACTH are mediated by cAMP and cAMP-dependent protein kinase, and depend on the synthesis of protein. The existence of adrenocortical steroidogenic regulatory proteins has been proposed, but none have yet been identified. The role of  $\text{Ca}^{2+}$  in ACTH action is complex;  $\text{Ca}^{2+}$ -regulated protein kinases may be involved. Pregnenolone, the product of the rate-limiting step in steroidogenesis, is poorly soluble in aqueous media and must be translocated between intracellular compartments; the translocation process is not understood. Furthermore, a biological role for pregnenolone, other than that of a precursor, has not been established. Progesterone, which is derived from pregnenolone, not only serves as a key steroid intermediate itself but is a ligand for a member of the steroid receptor gene superfamily. To examine the complex process of ACTH action, the possible involvement of specific steroid-binding proteins has been explored. A 34 kDa cytosolic pregnenolone-binding protein ( $\text{P}_5\text{BP}$ ) has been identified, purified and characterized. The  $\text{P}_5\text{BP}$  has multiple forms with  $\text{pI}$ 's of 6.5, 5.5, 5.4 and 5.2. Individual isoforms have been isolated, antisera generated and immunological identity demonstrated. Pregnenolone-binding activity is abolished by treatment with alkaline phosphatase. The  $\text{P}_5\text{BP}$  isoform patterns in the outer ACTH-responsive and inner ACTH-unresponsive zones are distinct and markedly altered by phosphatase treatment. The N-terminus of  $\text{P}_5\text{BP}$  is blocked, necessitating determination of internal peptide sequences after partial tryptic digestion.

These studies have shown that pregnenolone, the product of the rate-limiting step in steroidogenesis, binds specifically to a non-catalytic cytoplasmic protein ( $\text{P}_5\text{BP}$ ); furthermore, the binding-activity was found to be regulated by phosphorylation/dephosphorylation. The physiological role of  $\text{P}_5\text{BP}$ , although undoubtedly important, remains to be clarified. There are several possibilities: 1) the  $\text{P}_5\text{BP}$  may function as a transporter for pregnenolone, which is poorly soluble in an aqueous medium; 2) the  $\text{P}_5\text{BP}$  may act to regulate cholesterol side-chain cleavage (e.g., the dephosphorylated, pregnenolone-unbound form could suppress activity while the phosphorylated, pregnenolone-bound form relieves the inhibition or *vice*

versa; 3) the P<sub>5</sub>BP may be necessary for further metabolism of pregnenolone; 4) since pregnenolone can be metabolized by several routes (at least 6), perhaps the P<sub>5</sub>BP serves to ensure that pregnenolone is metabolized by a specific route; 5) pregnenolone may have a biological function in its own right, which is carried out when bound to a specific protein.

Progesterone, which is derived directly from pregnenolone, has been found to bind specifically to an acidic nuclear protein (P<sub>4</sub>BP) of M<sub>r</sub> 75-79 kDa; the binding activity appears not to be regulated by phosphorylation/dephosphorylation. The nuclear location of P<sub>4</sub>BP places the protein in the steroid receptor category, although the biochemical behavior of P<sub>4</sub>BP is different from that of the classical progesterone receptor, described in uterine and breast tissue, as well as the hen oviduct. As with P<sub>5</sub>BP, the physiological role of the P<sub>4</sub>BP remains to be elucidated. Perhaps the P<sub>5</sub>BP, which appears to be located primarily, if not solely, in the cytoplasmic compartment, is involved in the acute response to ACTH. In contrast, the P<sub>4</sub>BP, which appears to be located in the nucleus, is involved in the subacute response to ACTH. It is interesting to speculate as to whether both binding proteins are members of the steroid receptor gene superfamily.

The Section on Molecular Structure and Protein Chemistry, under **Hao-Chia Chen**, conducts research on the analysis, synthesis, and structure-function relationships of biologically active peptides and proteins. This includes the identification and synthesis of unusual structures and sequences in amino acids and peptides, and the development of new techniques for peptide sequencing and synthesis. Of particular interest are the structural design, chemical synthesis, and modification of molecules important to reproductive and developmental biology. A major component of this project focuses on the role of carbohydrate structures of human chorionic gonadotropin (hCG) in subunit association kinetics, and the effect of the carbohydrate structures on *in vivo* biological activity. Re-evaluation of previous studies on the reassociation kinetics of hCG subunits, measuring the fluorescence enhancement of 1-anilino-naphthalene-8-sulfonate that occurs with hCG subunit association, has revealed that the reassociation rate for deglycosylated subunits is 23-fold faster than that of the native hCG molecule. An initial time lag was noted during the reassociation. The fit of the data against four different models was tested and found to be most consistent with a model that predicts that the order of the reaction and the extent of lag depend on the concentration of the deglycosylated  $\alpha$ -subunit. The rate limiting step appears to be the conformational transition of the deglycosylated  $\alpha$ -subunit. Although a conformational transition of the  $\beta$ -subunit may also occur, the significance of this process as rate determining is minimal in all models tested. These findings have led to the proposal that hCG may be assembled as a non-deglycosylated form in living cells, and that the excess secretion of the carbohydrate-rich non-associable free hCG- $\alpha$ -subunit in disease states may be caused by over-glycosylation of the  $\alpha$ -subunit.

In related studies, HF-deglycosylated hCG has been purified and employed for studies of the pharmacokinetics and stimulation of testosterone production in the *Cynomolgus* monkey *in vivo*. These studies have shown that the removal of carbohydrate residues from hCG causes profound changes in the rate constant of hormone clearance, with no change in the initial volume of distribution. Deglycosylated hCG also remains a full agonist at the LH receptor in the primate *in vivo*, consistent with previous findings *in vitro*.

During the current year, structural design, chemical synthesis, conformational studies, and the mode of action of the vertebrate peptide antibiotics magainins have been studied. A major objective of this research is to elucidate the manner in which alpha-helicity, amphiphilicity, and stability to proteases contribute to broad spectrum antimicrobial activity without causing toxicity to mammalian tissues. Substitution in the natural magainin 2 sequence of residues Gly<sup>13</sup>, Gly<sup>18</sup> and Ser<sup>8</sup>, which have low propensity for helix formation, with Ala enhances  $\alpha$ -helical structure, and yields analogues with increases in antimicrobial activity of up to two orders of magnitude, but no appreciable increase in hemolytic activity compared with the natural magainins. Although more than 500 analogues have been synthesized by various laboratories, none surpasses the level of antimicrobial activity of these Ala-substituted analogues. Studies with eight magainin 2 amide analogues of D-Ala and

L-Ala permuted at positions 9, 13, 18 have revealed that D-Ala modification reduces  $\alpha$ -helical potential. In evaluating antimicrobial activity, modification at position 13 or 18 is less important than at 9, and the cumulative effect of a D-Ala modification at 9 and 13 is more deleterious than at 13 and 18. These results suggest that  $\alpha$ -helix in the N-terminal region is most important in eliciting biological activity, which is probably due to the presence of positive charges, which can interact with membrane phospholipids.

The ability of magainins to inhibit the growth of a wide variety of bacteria and to cause lysis of protozoa has prompted the suggestion that the peptides act upon lipid membranes. It has been proposed that magainins induce anion selective channels in black lipid membranes. However, current studies have shown that, in cytochrome oxidase lysosomes, magainin analogues dissipate membrane potential and release respiratory control, which requires proton translocation. In comparing the magainin 2 amide and a potent analogue, magainin A, both peptides were found to decrease the membrane potential of *E. coli* cells. However, magainin A causes a persistent drop in membrane potential, whereas magainin 2 amide is active only at much higher concentrations. Magainin A is also 40 times more active than magainin 2 amide in inhibiting the growth of *E. coli*. In cytochrome oxidase liposomes, magainin A is approximately 20% more effective than magainin 2 amide at a peptide concentration causing half-maximal stimulation of respiration. These results suggest that the difference in antimicrobial activity of these two peptides is not due simply to their ability to interact with lipid membranes. To examine the cause of this difference, the relative susceptibilities of magainin analogues to pronase inactivation in uncoupling the respiration rate of cytochrome oxidase were examined. Pronase is able to reverse the uncoupling caused by magainin 2 amide and to restore the coupled rate, but does not reverse the uncoupling activity of magainin A. This observation suggests that magainin A, which is a strong former of  $\alpha$ -helical structure, interacts with lipids much more readily than magainin 2 amide, and is thereby less susceptible to proteolysis. Such resistance to proteolysis may be in part responsible for the differences in antimicrobial activity of magainins, and for the increased biological activity of the potent analogues.

**Kao-Ping Huang**, who heads the Section on Metabolic Regulation, studies the role of protein kinases and phosphorylation-dephosphorylation of proteins in the regulation of cellular functions, and also the regulation and hormonal control of glycogen metabolism, and the activities of glycogen synthetase and phosphorylase kinase. The receptor-mediated turnover of membrane phospholipids plays an important role in the regulation of many cellular functions. Inositol 1,4,5-trisphosphate triggers the release of calcium from an intracellular, non-mitochondrial pool, whereas diacylglycerol activates PKC to modulate numerous cellular responses. This signal-transduction pathway has been implicated in the regulation of cell growth, differentiation, gene expression, hormone and neurotransmitter release, cell-surface receptor function, and cellular metabolism.

Phosphorylation-dephosphorylation of proteins is one of the most important mechanisms for the regulation of cellular functions. PKC, a  $\text{Ca}^{2+}$ /phospholipid-dependent protein kinase, has emerged as a pivotal regulatory element for cell growth, differentiation, gene expression, hormone secretion, cell surface receptor function, and cellular metabolism. This protein kinase can be activated by diacylglycerol, a second messenger generated by signal-induced breakdown of phosphoinositides. In addition, it has been identified as a receptor for tumor-promoting phorbol esters, which elicit pleiotropic responses comparable to those stimulated by many hormones and growth factors. Three major isozymic forms of PKC have been identified from mammalian brains. Polyclonal and monoclonal antibodies against these enzymes were prepared and utilized to determine their immunochemical characteristics and tissue distribution. These enzymes were found to have distinct tissue, cellular, and sub-cellular localizations, and to be differentially expressed during development.

Although PKC isozymes have been implicated in the regulation of diverse cellular functions, the role of each of these enzymes is unknown. One approach to investigate this problem is to identify a specific inhibitor or activator for each enzyme. Since PKC depends on  $\text{Ca}^{2+}$  and phospholipid for activity, the actions of these effectors on the kinase were investigated

by a "preincubation" protocol, to determine the resulting changes in kinase activity and phorbol ester binding. It was found that acidic phospholipids such as phosphatidylserine (PS), phosphatidic acid, phosphatidylglycerol, and cardiolipin can differentially inactivate PKC I, II, and III in the absence of divalent cations. The phospholipid-induced inactivation of PKC is concentration- and time-dependent and only affects the kinase activity without influencing the phorbol ester binding. PKC I is most susceptible to the phospholipid-induced inactivation and PKC III the least. Addition of the divalent cations,  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$ , suppresses the phospholipid-induced inactivation of PKC. In the absence of divalent cation, PKC I, II, and III all form complexes with PS vesicles, although to slightly different degrees, as analyzed by molecule sieve chromatography. [ $^3\text{H}$ ]phorbol ester bound to PKC I, II, and III can be recovered after chromatography; however, the kinase activities of all three enzymes are greatly reduced. In the presence of  $\text{Ca}^{2+}$ , all three PKCs form complexes with PS vesicles, and both the kinase and phorbol ester-binding activities of PKC II and III are recovered following chromatography. Under the same conditions, the phorbol ester binding activity of PKC I is also recovered, but the kinase activity is not. The phospholipid-induced inactivation of PKC apparently results from a direct interaction of phospholipid with the catalytic domain of PKC; this interaction can be suppressed by divalent cations. In the presence of divalent cations, PS interacts preferentially with the regulatory domain of PKC, resulting in activation of the kinase.

The structure/function relationships of PKC were analyzed by deletion analysis. PKC contains a regulatory domain in the  $\text{NH}_2$ -terminal half and a catalytic domain in the carboxyl-terminal half of the molecule. Within the regulatory domain there are two cysteine-rich  $\text{Zn}^{2+}$ -finger structures and a pseudosubstrate region. The regulatory domain was previously shown to contain phorbol ester and phospholipid binding activity. To define further the structure/function relationships, deletion analysis of the cDNAs was performed to determine the region of the PKC molecule important for conferring the regulatory function. The modified cDNAs were expressed in COS cells, and the products purified by column chromatography and analyzed by immunochemical and biochemical methods. Deletion of either cysteine-rich region has no effect on the binding of phorbol ester, whereas deletion of both cysteine-rich  $\text{Zn}^{2+}$ -finger regions eliminates the phorbol ester binding completely, indicating that the  $\text{Zn}^{2+}$ -finger structure is important for phorbol ester binding. Deletion of the phorbol ester-binding domain or the pseudosubstrate region, an area that presumably binds to the active site of PKC to keep the enzyme in an inactive form in the absence of activator, results in loss of the kinase activity. It was noted that the expression products of the modified cDNAs always associate with the particulate fraction, indicating that an unusual modification of the enzyme takes place during biosynthesis. It is possible that a unique "clearance" mechanism is functioning to convert the defective enzyme into a separate pool to prevent interference with the function of the intact kinase.

PKC inhibitors have been found to be very useful in defining the functional role of this enzyme in cellular regulation. Recently, it was demonstrated that a PKC inhibitor (H-7) can prevent HIV infection of cultured T-cells by inhibition of CD4 phosphorylation, a process believed to be essential for viral entry. Suramin, an anti-HIV reverse transcriptase agent, was found to exert biphasic effects on PKC; it activates these kinases at low concentrations ( $< 40 \mu\text{M}$ ), but inhibits them at higher concentrations. PKC I is most sensitive to activation by low concentrations of suramin and is most sensitive to inhibition by high concentrations of this agent. Among all the PKC inhibitors identified so far, suramin appears to be the first to display selectivity against the different isozymes. Since many trans-membrane signaling events act through PKC, it is likely that some of the functional specificity of cellular responses is transmitted by different PKC isozymes. This may also be true for the HIV infection process. Thus, the development of PKC isozyme-specific inhibitors should provide useful tools in the study of PKC-mediated processes and potential therapeutic agents.

Previous studies show that PKC isozymes (types I, II, and III) have distinct neuronal localizations in the cerebellum. The expression of these isozymes during the postnatal development of the rat cerebellum was followed by immunochemical analysis. By immunoblot analysis, type I PKC was found to be low within two weeks after birth, to show

an abrupt increase between two to three weeks, and level off afterward. By immunofluorescent staining, type I PKC-specific antibody recognizes the cell bodies and dendrites of Purkinje cells. The increase of this isozyme between two to three weeks of age correlates with the spreading of Purkinje cell arborization, at which time the bulk of synaptogenesis between dendritic spines and axons of granule cells occurs. Both type II and III PKCs are present in granule cells. At birth, the level of type II PKC is relatively high compared with that of type III PKC, and type II PKC-specific antibody stains the granule cell precursors in the external layer more heavily than does type III PKC-specific antibody. The level of type II PKC declines slightly after birth, increases again at one week and plateaus after three weeks, whereas the level of type III PKC increases gradually until leveling off after three weeks. Throughout development, type III PKC-specific antibody also stains the cell bodies of Purkinje cells, but not their dendrites. These results demonstrate that the developmental expression of each PKC isozyme is under separate control, and that their distinct cellular and subcellular localizations mirror their unique functions in the cerebellum.

As described above, both type II and III, but not type I, PKC isozymes are present in the granule cells of adult rat cerebellum. Type II and III PKCs, but not type I, are also present in primary cultures of granule cells prepared from 8-day old rats. However, the expression patterns of these isozymes are different. Except for a slightly elevated level at 4-5 days, type II PKC is expressed at a relatively stable level throughout 13 days in culture. By contrast, type III PKC is expressed at a low level initially and the content increases as the culture continues, up to 13 days. Treatment of the 8-day cultures, when type II PKC is the predominant species, with the phorbol ester PMA results in the down-regulation of PKC, whereas in the 13-day culture, when type III PKC is predominant, is less susceptible to down-regulation by PMA. Treatment of the cultures with glutamate, the excitatory amino acid, causes a reduction of both type II and III PKCs in the cytosol and an increase of these isozymes in the membrane fraction. Although the time course and extent of translocation varies from culture to culture, accumulation of type II, but not type III, PKC in a unique membranous fraction, which was not extractable by 0.5% NP40 containing buffer, was consistently observed. These results suggest that type II and III PKCs, with their dissimilar expression patterns and responses to glutamate, may also be endowed with dissimilar and specific functions in granule cells of cerebellum.

Phosphorylation of myosin light chain is thought to play an important role in the regulation of smooth muscle contraction. Phosphorylation of the light chain by myosin light chain kinase at Ser<sup>19</sup> and Thr<sup>18</sup> results in an increase in the actin-activated Mg-ATPase activity. In contrast, phosphorylation of the light chain at Ser<sup>1</sup>, Ser<sup>2</sup>, and Thr<sup>9</sup> by PKC counteracts the increase in the ATPase activity caused by myosin light chain kinase. In order to test the effect of proteolysis as a mechanism for the activation of PKC, the phosphorylation of myosin light chain by PKC was compared with that by the protease-degraded, spontaneously active enzyme, PKM. This study revealed that PKM phosphorylates the light chain to a higher stoichiometry than PKC. Analysis of the <sup>32</sup>P-labeled tryptic peptides derived from the light chain phosphorylated by PKM revealed that both the sites phosphorylated by PKC and myosin light chain kinase are phosphorylated by PKM alone. The PKM-mediated phosphorylation of both sets of phosphorylation sites is not affected by an inhibitor, ML-9, nor the activators of myosin light chain kinase, Ca<sup>2+</sup> and calmodulin. These results provide evidence that the substrate specificity of PKC can be altered following its conversion to PKM.

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## LABORATORIES OF THE SCIENTIFIC DIRECTOR

### Section on Viruses and Cellular Biology

Arthur S. Levine, M.D., Head



Left to right: M. Protic, A. Levine, K. Dixon, C. Patch

Under the direction of **Arthur Levine**, the Section is utilizing various probes to elucidate the molecular mechanisms of DNA replication, repair, and mutagenesis both in eukaryotic and prokaryotic cells.

Chromosomal mutations are the underlying cause of most inherited diseases and many developmental abnormalities. Mutations can also lead to alterations in gene expression in somatic cells, eventuating in the loss of the normal differentiated phenotype and ultimately, cellular transformation. In work within this Section directed by **Kathleen Dixon**, a shuttle vector, pZ189, has been developed which contains the SV40 replication origin and early region, so that it replicates like SV40 virus in primate cells. This vector also contains the pBR327 bacterial plasmid origin and replication functions, so that pZ189 can replicate in bacterial cells; the gene for ampicillin resistance to provide a selectable phenotype in bacterial cells; and the *supF* gene which codes for a suppressor tRNA and provides the target for mutagenesis. Thus, the vector (pZ189) allows one to induce mutations in the mammalian system, identify them in a bacterial system, and then sequence them. Both at the genetic and biochemical levels, the vector has proved very useful for investigating how DNA replication fidelity is maintained, how DNA damage caused by mutagenic agents is removed by cellular DNA repair processes, and how damage that remains in the DNA interferes with normal DNA replication and induces mutations. With this vector, mutations induced *in vivo* (cultured primate cells) by two different carcinogens, UV radiation and benzo[a]pyrene diolepoxide (BPDE), have been analyzed. Mathematical modeling and statistical analysis have been used to demonstrate that there is a good correlation between the sites and frequencies of UV-induced mutations and the sites and frequencies of UV-induced DNA dimers (in the *supF*

targets). In the case of BPDE-induced mutations, the mutational spectrum is consistent with the specificity of BPDE adduct formation. The dramatic differences in mutational specificity of these two carcinogens indicate that the observed mutations are a result of the specificity of the carcinogen used and not some unusual characteristic of the target DNA or the assay system. Additional work with the pZ189 vector system has demonstrated that inducible cellular responses (analogous to the bacterial "SOS" response to DNA damage) can influence mutagenesis in mammalian cells: This group has shown that when mammalian cells are treated with carcinogens prior to transfection with the UV-damaged vector, the frequency of UV-induced mutations increases; however, the types of UV-induced mutations are unchanged. Thus, there appear to be indirect as well as direct effects of DNA damaging agents on mutation frequency in mammalian cells, just as in bacteria. Enhanced mutagenesis can also be induced by the tumor promoter, phorbol myristate acetate.

The pZ189 vector system can also be used to study DNA replication fidelity and mutation fixation *in vitro*, with the vector being completely replicated in crude extracts prepared from monkey or human cells, which are supplemented with purified SV40 T-antigen. Previously, it was found that the fidelity of replication in this system is much higher than that seen in gap-filling reactions with purified DNA polymerases, indicating that there are additional factors in the crude system that serve to enhance replication fidelity. The role of these additional factors in maintaining fidelity was studied during the past year by comparing the fidelity of DNA synthesis catalyzed by DNA polymerase  $\alpha$  alone with that catalyzed by DNA polymerase  $\alpha$  in a DNA replication system containing five additional purified enzymes (DNA ligase, 5'→3' exonuclease, RNase H, topoisomerase II, and SV40 T-antigen). In the complete system DNA polymerase exhibits much higher fidelity than it does in the absence of accessory enzymes; thus, these accessory proteins appear to assist the polymerase in maintaining replication fidelity. Since the fidelity of the purified system is still somewhat lower than that of the crude *in vitro* system, there are undoubtedly additional factors present in the crude system that serve to further enhance replication fidelity, and fractionation of the crude system, now underway, should reveal the nature of these factors. The group has also begun to examine the replication of carcinogen damaged templates and mutagenesis of these templates in the crude *in vitro* system. These damaged templates become fully replicated in the *in vitro* system, as they do *in vivo*, and this replication is accompanied by mutation induction. Moreover, the types of base substitution mutations induced *in vitro* are similar to those induced *in vivo*. Overall, the *in vitro* DNA replication system appears to be a very powerful system in which to elucidate the molecular details of the process of mutation induction in mammalian cells.

This Section has expanded its studies on mechanisms of mutagenesis in mammalian cells to include rodent systems, so as to take advantage of the many well-characterized rodent somatic cell mutants with altered mutagenic phenotypes. Since the pZ189 shuttle vector replicates only in primate cells, it was necessary to construct an analogous polyoma virus-based vector that would replicate in a variety of rodent cells. This work has now been accomplished and one of the vectors so constructed has been used successfully in mouse lymphoma cells to analyze the difference in mutagenicity between DNA repair-deficient and repair-proficient lines. The repair deficient line is hypermutable by UV radiation, presumably due to its inability to excise UV photoproducts.

Miroslava Protic has previously shown that enhanced repair of UV-damaged SV40 expression vectors occurs in mammalian cells treated with carcinogens such as UV or mitomycin C. To determine the molecular basis for this enhancement, she has initiated *in vivo* and *in vitro* repair studies using DNA expression vectors and cells or cell-free extracts derived from cultured monkey and human cell lines. During the past year, she has shown that enhanced repair synthesis can be detected *in vitro* using expression vectors and extracts prepared from UV-treated monkey cells, implying that cellular factors responsible for the enhanced repair act on DNA substrates *in vitro* in a similar fashion as *in vivo*. Since some of these factors might be DNA lesion-recognition proteins, this group initiated DNA binding studies to identify such factors. Utilizing the DNA band-shift assay and specific DNA probes, they have now identified an inducible DNA binding protein that appears to be specific for UV-

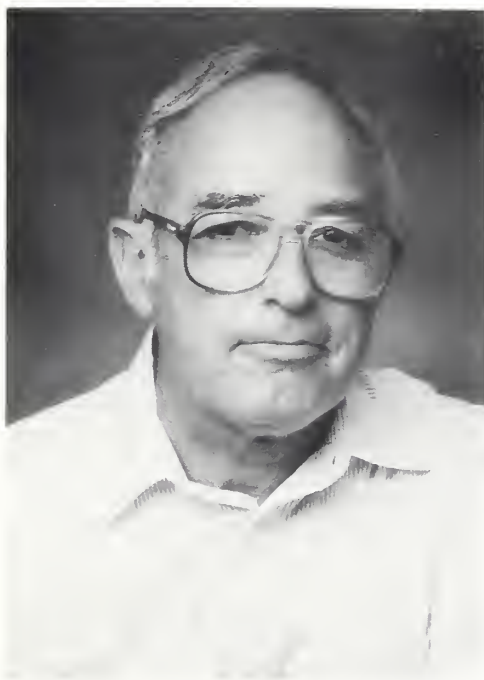
irradiated dsDNA. In UV treated monkey cells, they found a time and UV-dose dependent increase in the amount of this damage-specific DNA binding protein, with the increase totally abolished by the inhibitors actinomycin D and cycloheximide, suggesting a requirement for *de novo* protein synthesis. A similar increase in the UV-damage specific DNA binding protein (UV-DDB) was found in cells pretreated with the DNA polymerase inhibitor aphidicolin, suggesting that the inducing signal can either be damage to the cellular DNA or interference with cellular DNA replication. The most probable binding site for the UV-DDB protein is the (6-4) pyrimidine dimer. This DNA binding protein is the first such damage-specific, damage-inducible DNA binding protein identified in primate cells. The constitutive presence and inducibility of this protein in normal primate cells implies that it probably has a role in DNA repair and recovery of cells from the toxic effects of UV irradiation. Importantly, the group has recently found that the protein can neither be detected nor induced in cells from one of the cancer-prone XP complementation groups (group E), suggesting that the missing protein in these patients may account for their disease. Finally, during the past year the group began to purify the UV-DDB using monkey cell extracts. Three peaks of UV-DDB activity were detected in both mock- and UV-treated cells following ion exchange chromatography, and one of the three peaks was enhanced about 30-fold in pretreated cells. The native form of this inducible peak has a molecular weight of about 200 kD. During the coming year, efforts will be made to determine the structure and sequence of the UV-DDB protein; it is planned to synthesize complementary DNA oligonucleotides that can be used for detection of the UV-DDB genomic clone. This should ultimately permit the binding protein to be produced in sufficient amount to allow for purification to homogeneity; the purified protein can then be utilized to study the mechanism by which it recognizes a specific DNA lesion, binds to damaged DNA, and carries out its putative role in DNA repair.

**Cephas Patch** and other workers in the laboratory are studying the nature of molecular interactions between oncogene and anti-oncogene products that affect DNA replication—particularly as this balance influences cell differentiation and transformation. Since the oncogene alterations induced by several DNA viruses in model rodent systems and the oncogene changes found in some human tumors appear to be similar, rodent and human tumor systems are being studied in parallel. During the past year, an SV40 mutant (in the small t antigen gene) was investigated which, upon subcutaneous injection into newborn hamsters, induces osteosarcomas at a site distant from that of the injection. Wild-type virus only induces fibrosarcomas *in situ*. This result is provocative since it may shed light on the putative tissue-tropism function of small t. Moreover, it has recently been proposed that inactivation of the cellular "anti-oncogenes" *Rb* and *P53* by DNA virus early proteins might represent a common strategy used by these viruses to transform cells; the same "anti-oncogenes" are known to be altered in human osteosarcomas. The SV40-hamster osteosarcoma system may thus permit a better definition of the role of cellular anti-oncogenes in the regulation of DNA replication and in DNA virus tumorigenicity.



### Section on Growth Factors

**Gordon Guroff, Ph.D., Head**



Nerve growth factor, the major subject of the research in the Section on Growth Factors led by **Gordon Guroff**, was the first of the growth factors to be described. It is presently one of the best characterized of the several dozen growth and differentiation factors now recognized. These factors act on a wide variety of cell types in many different physiological settings. Nerve growth factor is required for the survival and differentiation of the sympathetic and sensory neurons of the peripheral nervous system. It has also been shown to act on the adrenal medulla, on certain tracts in the central nervous system, and on a number of different tumors. These latter two actions have sharpened the clinical interest in nerve growth factor, because they may indicate a role for nerve growth factor in degenerative diseases such as Alzheimer's and in certain malignancies. The biochemical mechanisms by which nerve growth factor acts are of substantial interest because an understanding of them may shed some light on fundamental processes of cell development in general and, specifically, on the growth and differentiation of neurons. There is also some data to indicate that nerve growth factor or nerve growth factor-like peptides enhance the repair and re-growth of damaged or severed neural tracts, a finding that would be of great clinical significance. A complete understanding of the way in which differentiation factors, such as nerve growth factor, limit the growth of normal cells promises to provide crucial insights into the growth regulation of normal cells and the tumors which arise from them. Finally, it is now clear that many of the growth factors and their receptors are structurally related to specific oncogenes, and that alterations in the biology of oncogenes are at the heart of the process by which cells become malignant.

The workers in this Section continue to study the intracellular mechanisms by which the signal generated by the combination of nerve growth factor with its receptor is mediated within the cell. The following advances have been made during the last year: (1) Nerve growth factor-induced changes in phosphoinositide turnover are accompanied by increased phosphorylation of a specific isozyme of phospholipase C; this observation provides a biochemical handle on what must be one of the earliest actions of nerve growth factor. (2) Nerve growth factor increases the secretion of catecholamines by PC12 cells and this

secretion is blocked by inhibitors of the L-type calcium channel; the involvement of the calcium channel provides an important insight into the linkage between the nerve growth factor receptor and calcium ion, a key second messenger. (3) A protein whose phosphorylation is decreased in cells treated with nerve growth factor, called Nsp100, has been identified as elongation factor 2 (EF-2), a participant in the cellular synthesis of protein; this identification specifies one exact role for nerve growth factor in the alterations in protein synthesis within differentiating cells. (4) The phosphorylation of the ribosomal protein S6 in cells treated with nerve growth factor, a differentiating agent, is on different sites than that in cells treated with epidermal growth factor, a mitogen; this finding indicates that the phosphorylation of the S6 protein plays a role in the alternate phenotypic routes, cell differentiation or cell division, that the cell can travel. (5) Differentiation of PC12 cells with nerve growth factor is accompanied by a decrease in the activity of the intracellular protease, calpain, caused, in turn, by an increase in the activity of its specific inhibitor calpastatin; the decreased activity of this protease for its preferred substrates in the cytoskeleton may be a key event in the morphological changes seen during nerve growth factor-induced neurite outgrowth. (6) PC12 cells infected with the *src* or the *ras* oncogenes undergo morphological changes similar to those seen upon nerve growth factor treatment, but neither set of differentiated cells executes the full range of biochemical differentiation that is seen with nerve growth factor; these findings suggest that while the corresponding protooncogenes may participate in nerve growth factor-induced differentiation, neither is a participant in the total chain of intracellular events. (7) Several of the actions of nerve growth factor on PC12 cells are more robust in cells near the G1/S border of the cell cycle; this observation opens the possibility that receptor levels or concentrations of intracellular mediators are keyed to the cell cycle.

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